MOLECULAR AND CELLULAR MECHANISMS REGULATING EXOCRINE PANCREAS GROWTH AND REGENERATION

by

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ABSTRACT

The pancreas is a complex organ consisting of both an endocrine and exocrine function. Cells of the endocrine pancreas control blood sugar, while the exocrine pancreas is responsible for providing enzymes used in digestion of food. These enzymes are synthesized by acinar cells, which comprise the majority of the organ. The work in this dissertation demonstrates that after birth, acinar cell proliferation, nuclearity, and ploidy are controlled by the signaling protein β-catenin as well as by novel intrinsic and extrinsic signaling factors, essential for postnatal and adaptive growth. This work establishes that β-catenin is acting not as a mitogen itself, but as a rheostat to tune the proliferative input from other sources. To test our rheostat model, we demonstrate that cholecystokinin (CCK)-dependent growth, an adaptive response to increased metabolic demand, requires β-catenin signaling to maximize acinar cell proliferation. We have also determined that mononucleate acinar cells preferentially contribute to early postnatal and regenerative growth, indicating the existence of subpopulations within the exocrine pancreas with distinct capacities for growth. Though binucleate acinar cells are initially inhibited from dividing, they regain the ability to divide in the adult organ. Though overall acinar cell proliferation in the adult organ is rare, the division of binucleate acinar cells produces a significant fraction of tetraploid, mononucleate acinar cells in the adult pancreas. When acinar cells

undergo regenerative proliferation in response to injury, however, cell cycle entry becomes restricted to mononucleate cells, recapitulating early postnatal expansion and protecting against the generation of aneuploid-vulnerable offspring. Taken together, this work demonstrates how the normal mass of acinar cells is attained and sustained in the mouse pancreas, as a genetic model for control of vertebrate organ size and a foundation for understanding diseases of the exocrine pancreas such as pancreatitis and pancreatic cancer.

I would like to dedicate this body of work to my mother, Pamela Jean Keefe, as a tribute to what she helped me begin to accomplish.

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The importance of family in all my life successes cannot be overstated. I am a reflection of their support, generosity, and perhaps most importantly, patience. I am extremely happy to include my wife, Alexandra Keefe, as a member of this happy bunch. Alexandra has been an incredibly important part of my graduate school journey and has made all aspects of my life more enjoyable with her company.

Though I have always been fascinated by scientific endeavors, as a young man, I considered them to be well beyond the scope of something I could achieve. I was obviously wrong in this assumption and for that, I have many people to thank. I was nearly led astray in my freshman year of college by a horrific chemistry teacher, who made the prospects of becoming a scientist seem unattainable. I was luckily able to transfer to another section where I met Susan Swope who literally saved my love for science and set me on the path towards becoming a scientist. After college, I was hired to my first job as a scientist, an opportunity that has opened many doors for me, and for that I would like to thank Nikolaus Trede as my first mentor. Early in my career, I met and worked with David Langenau and under his mentorship discovered an ethic and excitement for science that I have tried to adopt and make my own.

With a small amount of experience under my belt, I felt I knew what I wanted from a scientific advisor in graduate school. I wanted someone who was always around to bounce ideas off of and trouble-shoot protocols. I have seen what can happen when your advisor is unapproachable. I wanted someone who would allow me to enjoy life during my time as a graduate student and not an authoritarian who simply required my presence in the lab. I wanted someone who would compel me to work hard but support me in this process. I was lucky to land in Charlie Murtaugh's lab where quality of science and quality of life are both a priority. As my mentor, Charlie always set the bar high for success but more importantly, gave me the support to make success attainable.

CHAPTER 1

INTRODUCTION

Pancreatic organogenesis is a complex process that coordinates the interplay between intrinsic factors, such as the number of progenitor cells, and extrinsic signals that control the proliferation and fate of those progenitors. The relative contribution of these factors ultimately determines organ size. The vertebrate pancreas is comprised of three major cell types: endocrine islets, which secrete hormones that regulate glucose metabolism; and a network of exocrine acinar and duct cells, which are responsible for producing and transporting digestive enzymes, respectively. Acinar cells comprise the majority cell type in the pancreas and contribute to the bulk of pancreatic mass in the adult organ and thus have a significant bearing on final organ size. This thesis is focused on determining the molecular and cellular mechanisms that regulate exocrine pancreas size during growth and regeneration, with the hope that understanding these processes will generate insight into diseases, such as pancreatic cancer, in which organogenesis goes awry.

Developmental origins of the pancreas

Cells of the exocrine and endocrine pancreas are derived from common endodermal precursors that arise from a dorsal and ventral domain along the posterior foregut, at the end of gastrulation[1]. These cells are marked by expression of the homeobox transcription factor *Pdx1* (Pancreatic and duodenal homeobox 1)[1,2]. *Pdx1* is one of the earliest genes to be expressed in the developing pancreatic epithelium and is essential for normal organ formation[1,3]. The endoderm of both the dorsal and ventral *Pdx1*-expressing domains evaginates to form two epithelial buds encased in mesenchyme. All of the epithelial tissue of the pancreas will derive from these two endodermal buds. The mesenchyme surrounding these buds has been shown to enhance the proliferation of pancreatic endoderm cells, and later has an inhibitory effect on endocrine differentiation [4]. At E11.5, the pancreatic epithelium undergoes branching morphogenesis to produce a large pool of multipotent pancreatic progenitor cells (MPCs) marked by the expression of Carboxypeptidase 1 (*Cpa1*) [2,5]. The number of MPCs in the epithelium at this stage determines the eventual size of the mature pancreas [6]. Acini are derived from MPCs located at the periphery of the branching organ, with islets forming centrally [5]. By E15.5, the tip cells have become committed to an acinar fate and expansion of this lineage afterwards is predominantly through acinar cell replication rather than de novo formation of acini [1]. Lineage tracing studies have determined that once specified, acinar cell numbers are expanded by proliferation during embryonic, postnatal, and regenerative growth [7-10]. Chapter 2 of this dissertation focuses on whether

the embryonic requirement for b-catenin is recapitulated after birth for postnatal and regenerative growth[7,9-11].

Wnt/β-catenin signaling in the pancreas

The Wnts (*Wing*less and *I*NT-1) are a family of secreted glycoproteins that regulate a number of cellular functions throughout development and adult life. The Wnt family of ligands has been shown to activate three distinct pathways: the canonical Wnt/β-catenin signaling cascade, the noncanonical planar cell polarity pathway, and the Wnt/Ca^{2+} pathway. The focus of my research is centered on understanding the role of the canonical Wnt/β-catenin signaling pathway in pancreatic organogenesis. In the canonical pathway, Wnts transduce their signal by binding Frizzled and their co-receptors LRP5/6[12]. In the absence of Wnt stimulation, cytoplasmic β-catenin, a central player in the Wnt cascade, is phosphorylated by glycogen synthase kinase-3 (GSK-3) and targeted for ubiquitin-mediated degradation. The cytoplasmic regulation of β-catenin stability plays a key role in the signaling output of the canonical Wnt cascade. The binding of Wnt ligand to its receptors activates the intracellular protein Disheveled (Dsh), disrupting the destruction complex and allowing cytoplasmic β-catenin to accumulate, enter the nucleus, associate with the DNA binding partners LEF/TCF, and activate target genes[12].

The role of the Wnt/β-catenin pathway is complex and dynamically regulated during pancreas development. Previous studies showed that the conditional deletion of β-catenin in pancreatic progenitors resulted in a

hypoplastic pancreas due to the dramatic loss of exocrine acinar cells[7,9]. Subsequent studies have illustrated the divergent role for β-catenin in the developing pancreas, such that stabilization prior to E11.5 results in pancreatic hypoplasia, but after E13.5 increases pancreas organ size due to an increase in exocrine pancreas mass[10]. Significantly, these studies found that the manipulation of β-catenin did not lead to premature differentiation or death of progenitor cells, nor did it perturb islet cell mass or function; instead, β-catenin appears particularly critical for development of the exocrine lineage[7,13,14]. Recently, the deletion of β-catenin was found to reduce islet cell mass, partly through the loss of multipotent pancreatic progenitor cell (MPC) identity, ultimately leading to fewer progenitors and their progeny [11]. Taken together, these studies highlight the importance of this pathway in the maintenance of pancreatic progenitors early for the specification and expansion of the exocrine pancreas later in embryonic development. The role of the Wnt/β-catenin pathway in the postnatal pancreas is less clear. A significant portion of this thesis is dedicated to addressing the requirement for Wnt/β-catenin signaling in regulating acinar cell proliferation and identity during postnatal and regenerative growth of the pancreas.

Mouse models of pancreatitis and regeneration

In some types of cancer, inflammatory conditions are present before a malignant change, and are thought to be causal in the development of these cancers. The most common type of pancreatic cancer is pancreatic ductal

adenocarcinoma (PDAC), accounting for roughly 90% of pancreatic tumors[15]. The most recognized risk factor for developing the sporadic form of PDAC is chronic pancreatitis, which is triggered by acinar cell damage and followed by inflammation of surrounding pancreatic tissue[15]. Hereditary forms of pancreatitis have exposed a common mechanism by which pancreatitis can arise, whereby digestive proenzymes (zymogens) are prematurely activated within the cell, leading to damage and inflammation [15]. To date, almost all genetic factors found to increase the risk for pancreatitis are genes encoding pancreatic digestive zymogens or their inhibitors [16]. More common risk factors for developing pancreatitis in humans include alcohol abuse and gallstones[17]. Though the precise mechanisms by which these factors induce pancreatitis differ, they are both associated with the intrapancreatic activation of zymogens[17-19]. To recapitulate this injury, several animal models have been developed to study chronic and acute pancreatitis in the mouse, with the most well characterized being the administration of the cholecystokinin (CCK) ortholog, caerulein[16]. Similar to CCK, caerulein stimulates acinar cells to secrete digestive enzymes[20]. When caerulein is administered at supra-physiologic levels, it promotes excessive digestive enzyme secretion, ultimately leading to the improper activation of these zymogens within the cell[21]. Whether intracellular protease activation perfectly models human acute pancreatitis is not clear, but it has proved useful in understanding how the pancreas regenerates after this injury.

All organisms mount a biological response to damage, but vary widely in their ability to replace or regenerate tissues and organs. In contrast to steady-

state tissue turnover, regeneration is a process whereby new cells arise to replace those lost by injury. Humans and mice can regenerate injuries to the liver, pancreas, bone, and muscle, but are limited in their ability to repair damage to the heart and spinal cord. Regeneration relies on specific populations of progenitor that serve as the source of new cells in the regenerated tissues. These progenitors are considered stem cells with a limited potential, due to their unique ability to both self-renew and differentiate into lineage-restricted cell types [22]. But in the pancreas, it is mature differentiated cells that have the capacity to proliferate and produce new functionally specialized cells. The fact that mature differentiated cells can, under certain circumstances, undergo proliferation to replace damaged cells invokes a cellular plasticity that calls into question the classic use of the word progenitor. Simply put, replication of differentiated cell types is a viable possibility for normal tissue homeostasis and regeneration from injury and in the pancreas, we will refer to cells with this capacity as progenitors.

For bone and muscle, resident stem cells expand in number, differentiate, and contribute to regeneration and repair[23,24]. The regeneration process is different for the liver and pancreas, where mature differentiated cells of the organ reacquire the ability to proliferate and expand in number to replace damaged cells [8,25,26]. In the pancreas, acinar cells that are not damaged during injury transiently express genes associated with more primitive embryonic progenitor cells and reacquire the ability to proliferate and replace damaged tissue [8,27]. The regenerative capacity of acinar cells may be a double-edged sword, however, as cell cycle re-entry may put these cells at risk for hyperproliferative diseases, the

most devastating of which is pancreatic cancer. Our lab has shown that PDAC initiates among acinar cells and that initiation is accelerated by injury [28,29]. Another group has found acinar cells are much more susceptible to PDAC during the juvenile period, in which they are highly proliferative, than in the adult, when they are quiescent [30]. A significant portion of this thesis is focused on understanding the cellular and molecular mechanisms regulating acinar cell proliferation and maintenance of acinar cell identity during pancreas regeneration, to better understand how these factors are compromised in the stepwise progression towards tumorigenesis.

Diet-induced pancreas growth

Extrinsic signaling cues, like gut hormones released in response to the presence of food in the small intestine, have been shown to be essential for establishing pancreatic organ size, and yet the molecular mechanisms by which they control development are just beginning to be understood [20,31-33]. In the course of our investigation of acinar cell nuclearity, we noticed that the generation of binucleate cells coincided with the suckling-to-weaning transition in mice. During this transition, pups experiment with solid food and are exposed to a greater diversity of dietary protein, lipid, and carbohydrate than from their mother's milk. Previous studies have shown that the presence of protein and lipids in the small intestine triggers the secretion of cholecystokinin (CCK), a potent stimulant of acinar cell enzyme secretion [32]. CCK is also a powerful trophic hormone and has been shown to promote acinar cell hyperplasia and

hypertrophy after prolonged stimulation [33-35]. How prolonged CCK-stimulated secretion of digestive proenzymes ultimately promotes acinar cell proliferation is not known. Early studies using raw soya flour (RSF) found that pancreas size significantly increased with feeding and opened up the field of experimental pancreatic hyperplasia and neoplasia [36]. This was because high levels of a trypsin inhibitor found in RSF inhibits proteolysis in the small intestine, subsequently promoting increased CCK levels, driving acinar cell growth [36]. Camostat is a serine protease inhibitor with similar effects to RSF, and is widely used in modern studies to model pancreatic growth in response to increased dietary protein. Chapter 4 of this dissertation investigates whether CCK-dependent pancreatic growth requires β-catenin signaling.

Origins of acinar cell nuclearity and ploidy

Though mononucleate, diploid cells are widely considered the default for vertebrates, and a major role for binucleation and diploid-to-polyploid conversion has been described for normal development of cells within the liver, blood, and heart. The biological advantages of polyploidy have been proposed to include an improved response to metabolic demands and buffering against deleterious mutations that may precipitate tumorigenesis[37,38]. There are various mechanisms by which a cell could become bi- or multinucleate; including cell-cell fusion as occurs in skeletal muscle and osteoclasts, as well as incomplete cytokinesis as is seen in cardiomyocytes, trophoblast giant cells, and hepatocytes [24,39-42]. The rodent liver shares a common developmental origin with the pancreas, and thus

presents a likely candidate for the mechanism by which binucleate acinar cells arise. For hepatocytes, a mononucleate cell will undergo a round of DNA synthesis and abortive cytokinesis to produce a binucleate cell [40]. When a binucleate hepatocyte re-enters the cell cycle, it will produce tetraploid mononucleate daughter cells, further illustrating that in the liver, increased nuclearity and ploidy are tied to the cell cycle [40]. By comparison, a previous study looking at pancreatic acinar cell mitotic defects *in vitro* found naturally occurring binuclear and tetraploid mononuclear acinar cells, similar to hepatocytes [43]. Additionally, they found no evidence to implicate cell-cell fusion in the formation of binucleate cells, concluding that aberrant cytokinesis is the mechanism by which acinar cells generate binucleate cells [43]. How acinar cell nuclearity and ploidy are together regulated during postnatal growth and regeneration has not been determined and is the subject of Chapter 4 of this dissertation.

Mouse models of pancreatic development have provided important information about the diversity of function between mature cell types, derived from a common pancreatic progenitor, but have not identified any proliferative heterogeneity within a single lineage in the mature pancreas. Several studies looking for specialized progenitor cells capable of contributing to postnatal β-cell and acinar cell mass concluded that both these cell types grow by self-renewal and not by the proliferation of specialized progenitors [44-46]. However, what these studies could not discriminate between is the possibility of distinct populations of differentiated progenitors, within a restricted lineage in the adult pancreas.

After birth, acinar cells proliferate in large numbers to increase pancreatic mass. We found that as mice approach weaning, the number of cells undergoing DNA synthesis negatively correlates with an increase in pancreatic size, as the organ doubles in relative mass. The decrease in DNA synthesis at weaning has focused our attention on what factors could be influencing the cell cycle during this period of development, ultimately restricting organ growth.

Previous studies in the rat pancreas have described acinar cells as predominately mononucleate at birth, and progressively transitioning to >65% binucleate in the adult organ [47]. Although the number of binucleate cells is high, increased ploidy levels in rat acinar cells is low, suggesting that in the rat pancreas, a progression toward higher ploidy is arrested at the binucleate diploid DNA state (2 nuclei x 2n) [47]. A similar finding has been described for hepatocytes of the rat liver. This study looked at which factors had the strongest effect on ploidy, comparing the livers of 53 mammalian species, and found that postnatal growth rate highly correlated with hepatocyte ploidy[38,48]. Consistent with this argument, the growth rate in mouse liver is higher than in rat and, therefore, hepatocyte ploidy is higher in mouse [48,49]. Consistent with this argument, the growth rate of mouse pancreatic acinar cells has been shown to be higher than rat and, correspondingly, ploidy of mouse acinar cells is also higher. How binucleation and ploidy are together regulated in the pancreas has not been addressed. Somatic polyploidy has been described as a quick but perhaps dangerous solution for rapid organ growth, as it increases the amount of DNA template with which to make more protein, but in so doing produces a duplicate

set of centrosomes putting the cell at risk for aneuploidy if it divides again [38,48,50]. Whether distinct nuclearity and ploidy states of an acinar cell predispose them to malignant transformation is not known and is the focus of Chapter 3 of this dissertation.

Summary

How intrinsic and extrinsic factors are integrated to specify cell fates in combination with environmental stimuli is a central question in developmental and regenerative biology. Frequently, an organism re-uses the same signaling pathway to achieve unique developmental goals within different cellular contexts. We have defined the Wnt/β-catenin pathway as critical for the cellular response to intrinsic and extrinsic developmental cues that are necessary to drive organ growth during pancreatic organogenesis and regeneration. We have further identified a new developmental step in acinar cell development, which distinguishes mono- and binucleate acinar cells as functionally distinct subpopulations during postnatal and regenerative growth. Thus, we have identified both an intrinsic signaling component selectively important for acinar cell proliferation and the integration of this signal with extrinsic factors like CCK-signaling, to regulate nuclearity during postnatal and regenerative growth. It is my hope that this dissertation will contribute to a better understanding of the normal mechanisms controlling pancreatic organ size, and will prove useful to the growing understanding of the origin and nature of diseases that affect acinar cell proliferation.

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CHAPTER 2

β-CATENIN IS SELECTIVELY REQUIRED FOR THE EXPANSION AND REGENERATION OF MATURE PANCREATIC ACINAR CELLS IN MICE

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RESEARCH ARTICLE

β-catenin is selectively required for the expansion and regeneration of mature pancreatic acinar cells in mice

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SUMMARY

The size of the pancreas is determined by intrinsic factors, such as the number of progenitor cells, and by extrinsic signals that control the fate and proliferation of those progenitors. Both the exocrine and endocrine compartments of the pancreas undergo dramatic expansion after birth and are capable of at least partial regeneration following injury. Whether the expansion of these lineages relies on similar mechanisms is unknown. Although we have shown that the Wnt signaling component β-catenin is selectively required in mouse embryos for the generation of exocrine acinar cells, this protein has been ascribed various functions in the postnatal pancreas, including proliferation and regeneration of islet as well as acinar cells. To address whether β-catenin remains important for the maintenance and expansion of mature acinar cells, we have established a system to follow the behavior and fate of β-catenin-deficient cells during postnatal growth and regeneration in mice. We find that β-catenin is continuously required for the establishment and maintenance of acinar cell mass, extending from embryonic specification through juvenile and adult self-renewal and regeneration. This requirement is not shared with islet cells, which proliferate and function normally in the absence of β-catenin. These results make distinct predictions for the relative role of Wnt–β-catenin signaling in the etiology of human endocrine and exocrine disease. We suggest that loss of Wnt–β-catenin activity is unlikely to drive islet dysfunction, as occurs in type 2 diabetes, but that β-catenin is likely to promote human acinar cell proliferation following injury, and might therefore contribute to the resolution of acute or chronic pancreatitis.

INTRODUCTION

Of fundamental importance to developmental biology and medicine is the question of whether postnatal growth or regeneration of a tissue recapitulates the molecular mechanisms of its embryonic development. The answers are complex and variable across different tissues. For example, both bone formation and fracture repair require BMP-Smad signaling, although the relevant BMP ligands themselves differ between these processes (Rosen, 2009). By contrast, although the development of skeletal muscle progenitor cells requires *Pax3* and *Pax7*, these transcription factors are dispensable for regeneration by adult muscle stem cells (Lepper et al., 2009).

Addressing these questions in the pancreas, a bifunctional organ comprising anatomically distinct endocrine and exocrine cell types, has implications for several devastating diseases, including diabetes, pancreatitis and pancreatic cancer. Lineage-tracing studies in the mouse have recently converged upon the finding that, although endocrine islet cells are generated de novo in the embryonic pancreas, their postnatal maintenance and replenishment relies instead on the proliferation of differentiated cells (Dor et al., 2004; Kopinke and Murtaugh, 2010; Solar et al., 2009). With respect to mechanism, several genes that are dispensable for embryonic islet development but are required for the expansion of insulin-producing β-cells after birth have been identified in mice (Chen et al., 2009; Georgia and Bhushan, 2004; Kushner et al., 2005; Rane et al., 1999; Zhang et al., 2006). A further distinction between physiological and regenerative growth is suggested by the regeneration-specific β-cell defect observed in mice lacking the *Glp1r* gene (De Leon et al., 2003). Such a distinction might also apply to exocrine acinar cells, in which genes including *Notch1* and the hedgehog signaling component *Smo* are dispensable for normal development and homeostasis but are required for regeneration following caerulein-induced pancreatitis (Fendrich et al., 2008; Siveke et al., 2008). In the present study, we address the postnatal role of a gene that is required for acinar cell development, β-catenin (*Ctnnb1*) (Murtaugh et al., 2005; Wells et al., 2007).

Using the Cre-*loxP* system, we and others have previously shown that deletion of β-catenin, an essential component of the Wnt signaling pathway, abrogates acinar cell specification and development in mice (Murtaugh et al., 2005; Wells et al., 2007). We have further shown that this gene is dispensable for the survival and phenotypic maintenance of adult acinar cells, as well as for the function of adult insulin-producing β-cells (Murtaugh et al., 2005). Additional studies of Wnt–β-catenin signaling – using distinct methodologies – suggest a more complicated and context-dependent role for β-catenin. For example, other investigators have reported a pancreatitis-like phenotype in postnatal β-catenin knockout pancreata (Dessimoz et al., 2005; Morris et al., 2010; Wells et al., 2007), suggesting that this gene is required not only for differentiation of acinar cells but also for their normal maintenance. Depending on the experimental design, hyperactivation of Wnt–β-catenin signaling can cause pancreatic agenesis, acinar cell hyperplasia or islet cell hyperplasia (Heiser et al., 2006; Rulifson et al., 2007; Strom et al., 2007). Finally, inhibiting Wnt signaling in β-

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cells specifically is reported to impair their proliferation and glucose homeostasis function (Dabernat et al., 2009; Rulifson et al., 2007). These seemingly contradictory results suggest that the phenotype of β-catenin deletion in the pancreas is surprisingly dependent on the precise spatiotemporal domain of Cre activity.

With respect to the role of β-catenin in postnatal acinar cells, most previous studies have used Cre transgenes that are active throughout the developing pancreas, raising the possibility of non-cell-autonomous effects. By contrast, our own previous finding that β-catenin is dispensable in differentiated acinar cells was made in the uninjured adult pancreas (Murtaugh et al., 2005), where basal levels of proliferation and apoptosis are so low that alterations due to loss of β-catenin could go undetected. In the present study, we address the postnatal requirements for β-catenin under conditions of rapid as well as gradual cellular turnover and expansion. We find that β-catenin is indispensable for physiological and regenerative proliferation of acinar cells, but dispensable for their survival and phenotypic maintenance. Together, this work identifies a continuous requirement for β-catenin in acinar development, extending from specification in the embryo to self-renewal in the juvenile and regeneration in the adult.

RESULTS

Deletion of β-catenin in the adult pancreas does not compromise acinar cell maintenance

To establish a system in which the fate of β-catenin-deficient acinar cells could be directly and quantitatively compared to those of corresponding wild-type cells, we crossed mice carrying both a null allele of β-catenin and a deleter transgene, *Elastase-CreERT* (Murtaugh et al., 2005; Stanger et al., 2005) (*Ctnnb1*$^{\Delta/+}$; *ElaCreERT* mice), to mice that were double-homozygous for a conditional allele of β-catenin (Brault et al., 2001) and a Cre-dependent EYFP reporter construct (Srinivas et al., 2001) (*Ctnnb1*$^{lox/lox}$; *R26R*$^{EYFP/EYFP}$ mice). The *ElaCreERT*-containing offspring of this cross are genetically matched except for β-catenin: they are heterozygous either for the conditional and wild-type alleles (*Ctnnb1*$^{lox/+}$; *R26R*$^{EYFP/+}$; *ElaCreERT*) or for the conditional and null alleles (*Ctnnb1*$^{\Delta/lox}$; *R26R*$^{EYFP/+}$; *ElaCreERT*). The former genotype permits tamoxifen-inducible EYFP labeling of acinar cells without β-catenin deficiency and is henceforth referred to as 'control'. The latter permits simultaneous deletion of β-catenin and EYFP marking, and is henceforth referred to as 'ABKO', for acinar-specific β-catenin knockout. In principle, by comparing identically treated control and ABKO mice, we should detect a requirement for β-catenin in proliferation or survival as a relative decrease in the EYFP labeling indices of ABKO pancreata compared with those of controls.

To assess the functionality of this approach, we administered 10 mg tamoxifen to adult control or ABKO mice ('pulse') and analyzed EYFP labeling after 10 days ('chase'). As described in the Methods, we used quantitative immunofluorescence to compare the EYFP labeling index, between genotypes, of amylase-expressing acinar cells. We found widespread EYFP expression (>50%) by acinar cells of both control and ABKO mice (Fig. 1A,B), with indistinguishable labeling indices between the genotypes (Fig. 1C). Immunofluorescence confirmed that EYFP-positive (EYFP+) acinar cells of ABKO pancreata were negative for β-catenin (Fig. 1D-G), allowing us to use EYFP as a surrogate marker for the deletion of

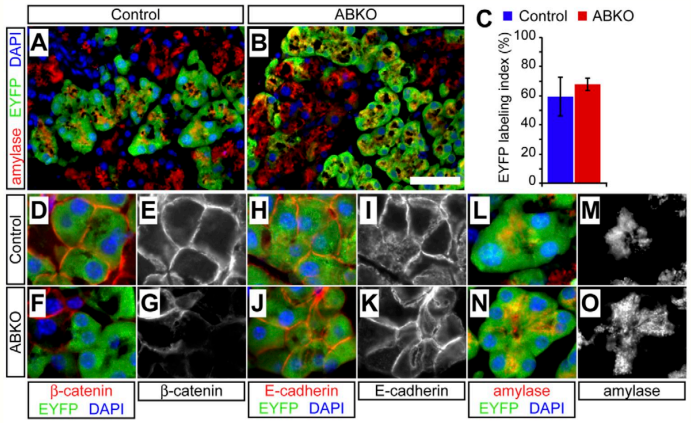

Fig. 1. Establishing a system to trace the fate of wild-type and β-catenin-deficient acinar cells. Control (*Ctnnb1*$^{lox/+}$; *R26R*$^{EYFP/+}$; *ElaCreERT*) and acinar-specific β-catenin knockout (ABKO: *Ctnnb1*$^{\Delta/lox}$; *R26R*$^{EYFP/+}$; *ElaCreERT*) mice received tamoxifen at 2 months of age and were analyzed 10 days later by immunofluorescence on frozen sections. (A,B) EYFP (green) is widely expressed in amylase+ acinar cells (red), indicating successful recombination. Scale bar: 100 μm. (C) Overall EYFP labeling of acinar cells does not differ between genotypes, 10 days post-tamoxifen (n=2 mice per genotype; P=0.48). (D-G) In controls, β-catenin localizes normally to the cell membrane of EYFP+ acinar cells, whereas, in ABKO pancreata, EYFP marks cells that have lost β-catenin expression. (H-K) E-cadherin exhibits identical membrane localization in EYFP+ acinar cells of control and ABKO mice, suggesting that loss of β-catenin does not perturb cell-cell adhesion. (L-O) Expression and apical localization of amylase is identical in control and β-catenin-deficient cells, indicating that loss of β-catenin does not affect acinar polarity or differentiation state.

β-catenin. Pancreata from control and ABKO mice that did not receive tamoxifen [harvested at postnatal day 60 (P60)] exhibited a low amount of acinar cell EYFP labeling [control (n=3): 4.8±2.3%; ABKO (n=3): 4.0±0.8%; P=0.8], resulting from leakiness of the *ElaCreERT* transgene. As expected from our previous work (Murtaugh et al., 2005), EYFP-labeled ABKO acinar cells were outwardly indistinguishable from controls, with normal expression of the epithelial junction protein E-cadherin (Fig. 1H-K) and normal polarity, as indicated by the apical localization of amylase-containing secretory granules (Fig. 1L-O).

These results indicate that our experimental design can be used to compare acinar cell maintenance between genotypes, in terms of both number and phenotype. They also confirm that β-catenin is dispensable for the survival of acinar cells and the maintenance of their differentiated state, contrasting with its essential requirement during development (Murtaugh et al., 2005).

β-catenin-deficient acinar cells do not contribute to postnatal expansion of the exocrine compartment

The adult mouse pancreas is a highly quiescent organ, and very few acinar cells would be expected to divide during the course of the preceding experiment (≤2% per day) (Magami et al., 2002; Teta et al., 2007). Acinar cells divide much more rapidly shortly after birth, as the entire organism grows in mass and cellularity (Magami et al., 2002), and we sought to investigate the role of β-catenin signaling during this expansion phase. To this end, we performed neonatal knockout experiments by administering tamoxifen (10 mg) to nursing mothers of newborn (P0) control and ABKO pups (Kopinke and Murtaugh, 2010), and analyzed the distribution of EYFP label in P7 infants and P30 juveniles.

The pancreata of neonatal ABKO mice were noticeably smaller than those of controls at P7 (Fig. 2A,B) but not at P30 (data not shown). This might indicate that loss of β-catenin affects acinar cell expansion only during the first few days of life, or else that compensatory growth of unrecombined acinar cells eventually makes up for the deficiency imposed by β-catenin deletion. The latter possibility was supported by quantification of EYFP-labeled acinar cells, which revealed a twofold reduction in labeled ABKO acinar cells at P7, compared with control littermates (Fig. 2C,D), increasing to a fivefold reduction by P30 (Fig. 2E-G). At the latter stage, most acinar cells in ABKO pancreata remained EYFP negative and retained β-catenin expression (Fig. 3A,B). These data indicate that β-catenin function is required for the normal expansion of postnatal acinar cell mass.

The relative deficiency of EYFP+ acinar cells, in the ABKO background, could be caused by any of several cellular defects, including loss of differentiation state, increased cell death and decreased proliferation. Although, under normal physiological conditions, adult acinar cells do not transdifferentiate into duct or islet cells (Desai et al., 2007; Strobel et al., 2007), they can adopt a duct-like phenotype during cell culture (De Lisle and Logsdon, 1990; Means et al., 2005), and in response to oncogenic and mitogenic signals in vivo (Blaine et al., 2010; De La O et al., 2008; Habbe et al., 2008; Ji et al., 2009; Morris et al., 2010). However, this did not seem to be the case for neonatal acinar cells that had lost β-catenin: as was found in the short-term adult labeling experiment described above (Fig. 1), EYFP expression in P30 juvenile knockout pancreata identified cells that lacked β-catenin but retained normal

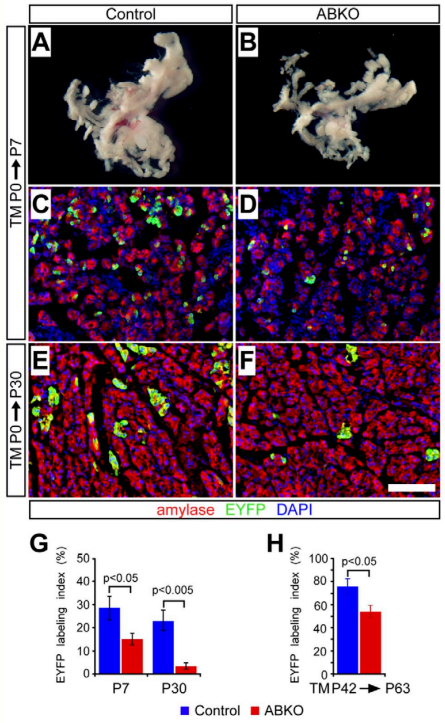

Fig. 2. β-catenin is required for physiological expansion of acinar cells. Control or ABKO mice received tamoxifen (TM) either on the day of birth, via maternal gavage, or as adults (P42), and were analyzed as neonates (P7), juveniles (P30) and adults (P63) (n=4-7 mice per genotype per time point). (A,B) At P7, pancreata of TM-treated ABKO mice are visibly smaller than those of control littermates (photographs taken at identical magnification). (C-F) Staining for EYFP lineage labeling (green) of amylase+ acinar cells (red) reveals relatively fewer EYFP+ cells in ABKO pancreata at P7 (C,D) and P30 (E,F), compared with controls. Scale bar: 100 µm. (G) The EYFP labeling index of amylase+ acinar cells is decreased at both time points in young ABKO mice, indicating that β-catenin-deficient acinar cells do not efficiently contribute to postnatal expansion of the exocrine pancreas. (H) Control and ABKO mice received tamoxifen at 6 weeks of age (P42) and were assayed for EYFP expression after a 3-week chase (P63). The EYFP labeling index of amylase+ acinar cells is decreased in ABKO pancreata, consistent with a requirement for β-catenin in the slow homeostatic expansion of acinar cells.

expression of the acinar marker amylase (Fig. 3A-D). Furthermore, although *ElaCreERT* induces a low level of ectopic recombination in insulin+ β-cells (Strobel et al., 2007), we found no significant difference in the fraction of EYFP-labeled β-cells between control and ABKO pancreata [control (n=2): 3.4±0.3% EYFP labeling of insulin+ cells; ABKO (n=2): 5.1±0.4% labeling of insulin+ cells; P=0.09], suggesting that β-catenin-deficient acinar cells had not transdifferentiated to islets (Fig. 3E,F). Finally, EYFP+ cells did not

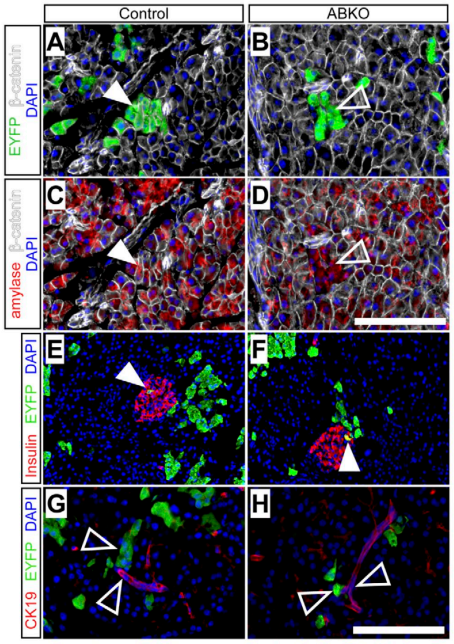

Fig. 3. Acinar cells maintain normal differentiation status in the absence of β-catenin. Control or ABKO mice received tamoxifen at birth and were analyzed as juveniles (P30). (A,B) At P30, EYFP+ cells (green) co-express β-catenin (white) in control but not ABKO pancreata (closed and open arrowheads, respectively). (C,D) Both control and β-catenin-deficient ABKO pancreata maintain normal cytoplasmic amylase expression (red). (E,F) Leaky recombination within insulin+ β-cells (red) by *ElaCreERT* produces similar islet labeling in control and ABKO pancreata (arrowheads). (G,H) EYFP+ cells of control and ABKO pancreata do not co-express the duct marker CK19 (red). Arrowheads indicate adjacent, non-overlapping EYFP+ and CK19+ cells. Scale bars: 100 μm.

express the ductal marker CK19 in either control or ABKO backgrounds (Fig. 3G,H). These data indicate that, although β-catenin is needed for acinar development in utero, its deletion after birth does not divert acinar cells to an alternative fate, but instead prevents their normal expansion during juvenile growth.

β-catenin is required for proliferation of neonatal acinar but not islet cells

To determine whether the requirement for β-catenin in acinar expansion was manifested at the level of cell division, we compared control and β-catenin-deficient acinar cells for expression of the proliferation marker Ki67. Immunostaining revealed a fourfold relative decrease, compared with controls, in the fraction of EYFP-labeled acinar cells staining for Ki67 in ABKO pancreata at P7 (Fig. 4). Although a compensatory increase in proliferation might be expected among those acinar cells that retain β-catenin in the ABKO pancreas, we found no significant change in the Ki67+ fraction of EYFP-negative (EYFP–) acinar cells between control (30.3±2.6%; n=5) and ABKO (30.5±1.4%; n=7; P=0.9) pancreata at this stage. It will be interesting to determine whether β-catenin-retaining acinar cells of ABKO pancreata exhibit a higher proliferation index at later stages, when overall proliferation declines in controls. In contrast to the dramatic proliferation phenotype of β-catenin-deficient cells, we found that EYFP+ acinar cells in ABKO pancreata were not subject to increased rates of apoptosis when compared with controls, as indicated by a similar (and very low) proportion of EYFP+ acinar cells co-expressing the apoptosis marker cleaved caspase-3 (cCasp3) at P7 [control (n=2): 0.5±0.05%; ABKO (n=2): 0.7±0.4%; P=0.7]. Because decreased cell division, even without increased death, would suffice to explain the impaired expansion of ABKO cells, we conclude that proliferation represents the major requirement for β-catenin in neonatal acinar cells.

Although β-catenin is needed to generate acinar cells in the embryonic pancreas, it is dispensable for islet cell specification (Murtaugh et al., 2005). As noted in the Introduction, however, various studies have suggested a role for Wnt–β-catenin in postnatal islet growth or function (Dabernat et al., 2009; Liu and Habener, 2008; Rulifson et al., 2007). To determine whether islet and acinar cells share a requirement for β-catenin in proliferation, we deleted β-catenin in endocrine precursors specifically using a neurogenin-3 (*Ngn3*)-*Cre* BAC transgene (Schonhoff et al., 2004). The resulting islet-specific β-catenin knockout (IsBKO) mice were recovered at weaning in normal mendelian ratios, and exhibited neither impaired survival nor glucose intolerance through ≥1 year of age (supplementary material Fig. S1). We analyzed control and IsBKO pancreata for proliferation at P7, when islet as well as acinar cells undergo rapid proliferative expansion (Magami et al., 2002). Staining of IsBKO pancreata at this stage revealed normal organization of insulin+ β-cells and glucagon+ α-cells into islets devoid of β-catenin protein (Fig. 5A-H). Furthermore, β-catenin-deficient islet cells proliferated indistinguishably from controls, as indicated by BrdU labeling (Fig. 5I-K). These results support our prior finding that pan-pancreatic β-catenin deletion did not impair adult islet function (Murtaugh et al., 2005) and suggest that, as is the case in utero, the major functional requirement for β-catenin after birth is specific to acinar cells. This specificity also indicates that β-catenin does not represent a 'housekeeping gene' for cell cycle progression that is required in all cell types.

β-catenin is required for homeostatic proliferation of acinar cells in the adult pancreas

Whereas the juvenile pancreas grows rapidly, in parallel with the organism, the adult pancreas grows slowly and exhibits little turnover. Nonetheless, rare division of acinar cells can be observed in the adult (Magami et al., 2002; Teta et al., 2007), raising the question of whether this low level of proliferation also depends on β-catenin function. To determine whether β-catenin is required for acinar cell proliferation in the normal adult pancreas, we pulsed control and ABKO mice with tamoxifen at 6 weeks of age and assayed EYFP expression and proliferation at 9 weeks of age. To maximize the detection of rare S-phase events, we administered BrdU via the drinking water for 7 days prior to sacrifice, thereby capturing a week of cell proliferation in the adult pancreas (Teta et al., 2007). We found a modest but significant decrease in the fraction of EYFP+ acinar

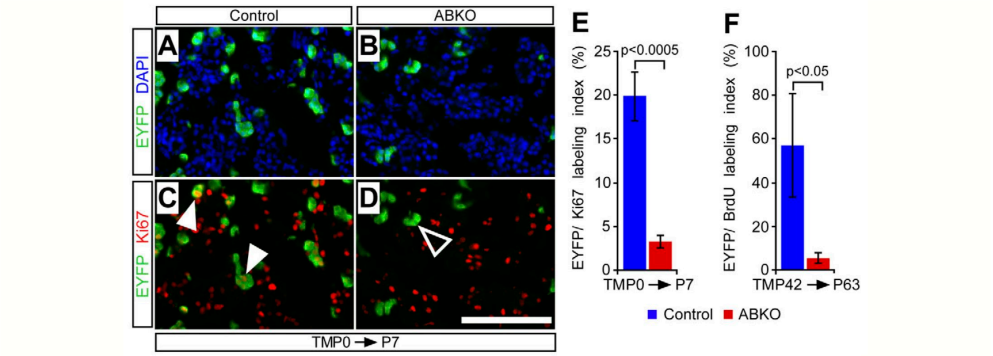

Fig. 4. Neonatal and adult acinar cell proliferation requires intact β-catenin function. To determine whether β-catenin is required for acinar cell proliferation in the neonatal and adult pancreas, we pulsed mice with tamoxifen on the day of birth, via maternal gavage, or as adults (P42), and analyzed neonatal (P7) and adult (P63) pancreata, respectively. Prior to sacrifice, adult mice received a 7-day BrdU pulse to label proliferating cells. (A,B) Staining for EYFP+ acinar cells (green) reveals a reduction in β-catenin-deficient acini in ABKO pancreata. (C,D) The fraction of proliferating EYFP+ acinar cells, marked by anti-Ki67 antibody labeling (white arrowheads), is markedly decreased in ABKO mice (open arrowhead indicates Ki67-negative/EYFP+ acinar cells) compared with controls. Scale bar: 100 μm. (E) Quantification of the Ki67 labeling index of EYFP+ cells reveals a significant reduction in the proliferative capacity of β-catenin-deficient acini compared with controls (n=5-7 mice per genotype). (F) Staining for BrdU revealed an approximately tenfold reduction in the fraction of BrdU-labeled EYFP+ cells in ABKO mice compared with controls, indicating a loss of proliferative ability in the resting adult pancreas (n=4-5 mice per genotype).

cells in ABKO pancreata compared with controls (Fig. 2H), suggesting that loss of β-catenin prevents normal expansion of acinar cells. More dramatically, staining for BrdU revealed a tenfold reduction in the fraction of BrdU+ EYFP+ β-catenin-deficient acinar cells compared with controls (Fig. 4F). Thus, we conclude that β-catenin is required not only for rapid acinar expansion in the juvenile pancreas, but also for steady-state proliferation in the adult.

Loss of β-catenin does not sensitize adult acinar cells to pancreatitis-associated injury

The pancreas is capable of at least partial regeneration from a variety of injuries, including acute pancreatitis induced by supraphysiological levels of the acinar secretagogue caerulein (Willemer et al., 1992). Regeneration in this model is preceded by transient expression of progenitor-like markers, including Pdx1

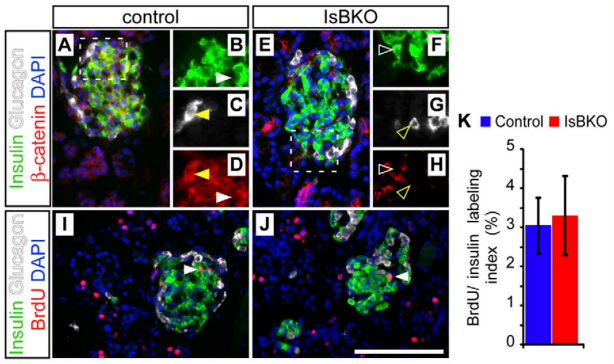

Fig. 5. Islet cells develop and proliferate independently of β-catenin status. Neonatal (P7) control (*Ctnnb1*$^{lox/+}$*; Ngn3-Cre*) and islet-cell-specific β-catenin knockout (IsBKO: *Ctnnb1*$^{\Delta/lox}$*; Ngn3-Cre*) mice were administered BrdU 1 hour prior to sacrifice and analyzed by immunofluorescence. (A-D) Islets of control mice exhibit clear β-catenin staining (red) in both insulin+ β-cells (green) and glucagon+ α-cells (white). Dashed lines in A indicate the area magnified in B-D. White and yellow arrowheads indicate insulin+ and glucagon+ cells, respectively, expressing β-catenin (closed; B-D) or lacking β-catenin (open; F-H). (E-H) Islet morphology and marker expression is normal in IsBKO pancreata, despite loss of β-catenin in β- and α-cells. (I,J) Anti-BrdU staining (red) reveals a similar distribution of proliferating cells among control and IsBKO endocrine cells. Arrowheads denote BrdU+/insulin+ β-cells. (K) Quantification reveals no significant difference in BrdU labeling of β-cells between control and IsBKO mice (n=2 mice per genotype; P=0.97). Scale bar: 100 μm.

(Fendrich et al., 2008; Jensen et al., 2005; Morris et al., 2010), suggesting that re-establishment of acinar cell mass involves a genetic program similar to that of embryonic acinar development. Indeed, it is reported that pancreas-specific β-catenin knockout mice (generated using a *Ptf1a*^{Cre} deleter strain) exhibit an almost complete loss of acinar cells after caerulein treatment (Morris et al., 2010). To determine the cell-autonomous requirement for β-catenin during injury and regeneration, we induced a pulse of acinar-specific recombination by tamoxifen treatment (10 mg) of adult control and ABKO mice, waited 1 week, and induced pancreatitis by a 2-day course of caerulein treatment (Jensen et al., 2005).

Whereas saline-treated pancreata appeared normal at all time points, pancreata of mice that were administered caerulein exhibited clear abnormalities at 2 days post-treatment, including fibroblast and leukocyte infiltration (Fig. 6A-D), and acinar-ductal metaplasia indicated by co-expression of amylase with the duct marker CK19 (supplementary material Fig. S2). Previous studies have demonstrated that acinar cells undergoing pancreatitis-induced metaplasia upregulate Pdx1, a transcription factor normally expressed in progenitor cells, while maintaining expression of Ptf1a, a master regulator of acinar cell identity (Jensen et al., 2005; Molero et al., 2007). Both of these phenomena were observed identically between control and β-catenin-deficient acinar cells, confirming that loss of β-catenin does not disrupt the differentiation state of regenerating acinar cells (supplementary material Fig. S3). Importantly, there was also no difference in the fraction of EYFP+ acinar cells between ABKO and control mice at the 2-day post-caerulein time point, indicating that loss of β-catenin did not affect the ability of acinar cells to survive pancreatitis-associated injury (Fig. 6E-I).

As an independent indicator of pancreatic injury, we assayed serum amylase levels before and after treatment, and found that caerulein-treated mice of both genotypes exhibited a transient increase in serum amylase 1 hour after the last caerulein injection, with a return to baseline 1 day later (supplementary material Fig. S4). Finally, staining for the apoptotic marker cCasp3 revealed quantitatively similar overlap with EYFP+ acinar cells in control and ABKO pancreata, indicating that pancreatitis-associated injury was not more likely to trigger apoptosis of β-catenin-deficient acinar cells than in control cells (Fig. 6J-L). Taken together, our results indicate that pancreatitis is not aggravated by deletion of β-catenin specifically in acinar cells, in contrast to a previous study of mice with pan-pancreatic β-catenin deficiency (Morris et al., 2010).

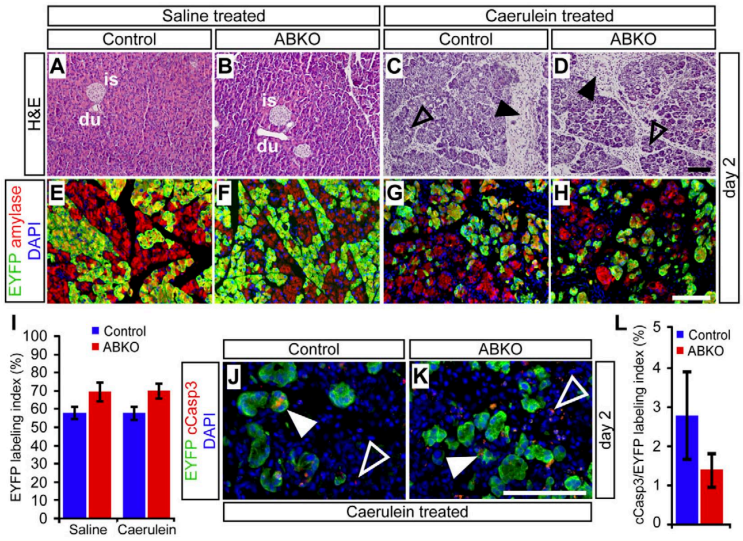

Fig. 6. β-catenin-deficient acinar cells are not preferentially injured during caerulein-induced pancreatitis. Control and ABKO mice received tamoxifen at 2 months of age and were treated with caerulein 1 week later to induce acute pancreatitis. The extent of acinar cell regeneration was analyzed histologically on wax sections, and quantitatively by immunofluorescence on frozen sections. (A-D) 2 days after the injection series was complete, H&E staining revealed no abnormalities in the pancreata of saline-treated mice, and a similar appearance of overall injury between caerulein-treated control and ABKO mice. At this stage, acute pancreatitis is manifested by infiltrating fibroblasts and inflammatory cells (black arrowheads) and the presence of dilated, metaplastic acini (open arrowheads). is, islet; du, duct. (E-H) A similar extent and distribution of EYFP labeling (green) is observed among amylase+ acinar cells (red) of saline- and caerulein-treated mice, regardless of genotype. (I) Quantification reveals no significant difference in the percentage of *R26R*^{EYFP}-labeled acinar cells, between treatments or genotypes (n=4-5 mice per genotype per treatment group). (J-L) At 2 days after caerulein treatment, double-staining for EYFP (green) and the apoptosis marker cleaved caspase-3 (red) reveals no increase in apoptosis among *R26R*^{EYFP}-labeled acinar cells of control and ABKO mice (n=3 mice per genotype). White arrowheads indicate cCasp3+ cells, co-expressing EYFP (closed) or unlabeled (open). Scale bars: 100 μm.

β-catenin is required for acinar cell proliferation during regeneration

Previous studies have found substantially complete acinar regeneration within 7 days following caerulein treatment (Fendrich et al., 2008; Jensen et al., 2005). To look specifically at regeneration, we analyzed control and ABKO pancreata 14 days after saline or caerulein administration, from mice treated in parallel to those analyzed at the 2-day time point. The appearance of these pancreata was grossly indistinguishable between genotype and treatment groups, and the histology of 14-day post-caerulein ABKO pancreata was similar to that of caerulein-treated controls (Fig. 7A-D). This result differs from the almost complete involution and fatty replacement of acinar tissue found after caerulein treatment of mice with a pan-pancreatic deletion of β-catenin (using *Ptf1a*^{Cre}) (Morris et al., 2010).

Examination of EYFP labeling at the 14-day time point, however, revealed a dramatic decrease in the labeling index of ABKO acinar cells following regeneration (Fig. 7E-I). As in the neonatal labeling experiments, residual EYFP+ acinar cells were β-catenin deficient, yet exhibited a normal differentiated acinar phenotype (supplementary material Fig. S5).

In both genotypes, normalization of organ morphology was accompanied by resolution of metaplasia, as indicated by downregulation of the duct marker CK19 in EYFP+ acinar cells (supplementary material Fig. S2). As noted above, β-catenin-deficient and control acinar cells exhibited similar levels of apoptosis following caerulein administration, suggesting that cell death did not prevent mutant cells from contributing to regeneration. Instead, we found a major defect in proliferation: administering BrdU 1 hour prior to sacrifice revealed a dramatic decrease in S-phase cells among EYFP+/β-catenin-deficient acinar cells at 2 days post-caerulein treatment (Fig. 7J-L). As in juvenile mice, we did not observe evidence for a compensatory increase in the proliferation index of EYFP-negative acinar cells [control + caerulein (n=4): 1.8±0.26%; ABKO + caerulein (n=4): 1.6±0.35%; P=0.6]. The fraction of wild-type acinar cells incorporating BrdU was relatively low at this early time point, leaving open the possibility that an additional defect in survival, manifested at an intermediate time point not yet analyzed, could have contributed to the ultimate decrease in ABKO acinar cells at 14 days post-caerulein. Alternatively, the window of wild-type acinar cell proliferation might be extended when a substantial fraction of the pancreas comprises ABKO cells, such that even a relatively low wild-type proliferation rate could produce dramatic out-competition of ABKO cells. In any event, the overall agreement between these results and our findings in neonatal and uninjured adult mice indicate a continuous requirement for β-catenin in acinar cell proliferation, during both physiological and regenerative growth.

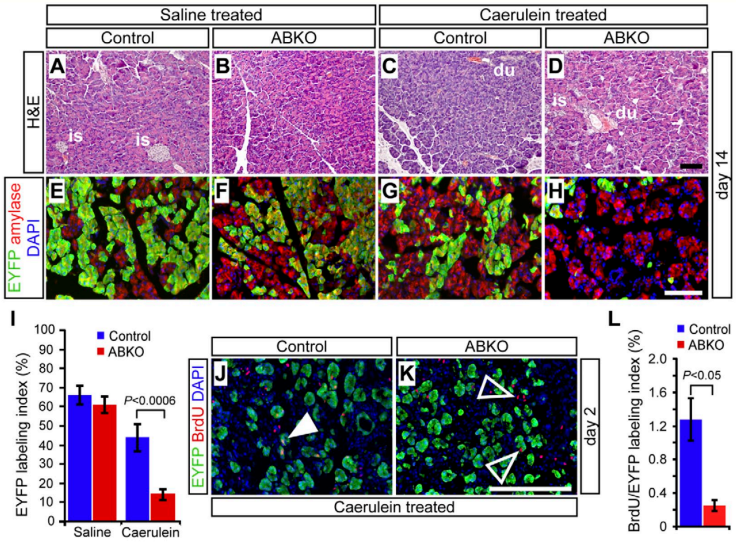

Fig. 7. β-catenin is required for regenerative expansion of acinar cells. (A-D) 14 days after treatment with saline or caerulein, H&E staining reveals comparable regeneration and resolution of fibrosis in control and ABKO mice subjected to acute pancreatitis. is, islet; du, duct. (E-I) EYFP labeling (green) is similar between saline and caerulein-treated control mice, whereas the fraction of EYFP+ acinar cells is dramatically reduced in ABKO pancreata (n=5-7 mice per genotype and treatment group; P<0.0006). (J-L) Control and ABKO mice were administered a 1-hour BrdU pulse, at 2 days post-caerulein treatment, sacrificed and analyzed for labeling. The fraction of BrdU+ *R26R*^{EYFP}-labeled cells is significantly reduced in ABKO pancreata, indicating decreased acinar cell proliferation following acute pancreatitis (n=3 mice per genotype; P<0.05). Scale bars: 100 μm.

DISCUSSION

During postnatal growth and regeneration of the pancreas, new acinar cells are generated almost exclusively from the division of pre-existing acinar cells (Desai et al., 2007; Strobel et al., 2007). β-catenin has an established role in the embryonic development of acinar cells (Murtaugh et al., 2005; Wells et al., 2007); however, whether this requirement applies after birth has not been addressed. Here we demonstrate a continuous requirement for β-catenin in the establishment and maintenance of acinar cell mass, extending from organogenesis in the embryo through expansion and homeostasis in the juvenile and adult organ, as well as regeneration following injury.

To our knowledge, this is the first study to interrogate the specific requirement for β-catenin in adult acinar cells. In several respects, our findings extend those of others and ourselves. First, we and others have shown that β-catenin deletion during organogenesis reduces acinar cell number at birth and beyond (Morris et al., 2010; Murtaugh et al., 2005; Wells et al., 2007). Here, we demonstrate a continued requirement for β-catenin after birth, operating at the level of proliferation rather than specification or differentiation. Recent studies indicate that acinar-like 'tip cells' of the embryonic pancreas represent multipotent progenitors that contribute to islets and ducts for the first several days of pancreatic organogenesis (Zhou et al., 2007). It is tempting to speculate that immature tip cells also require β-catenin for proliferative expansion, the failure of which partly explains the embryonic β-catenin deletion phenotype.

As well as acinar cells, we investigated the role of β-catenin in postnatal islet cells; none was detectable. This result agrees with prior studies of adult, pan-pancreatic β-catenin knockout mice, which exhibit normal glucose homeostasis (Dessimoz et al., 2005; Murtaugh et al., 2005; Wells et al., 2007), but contradicts the emerging hypothesis that β-catenin promotes proliferation or function of insulin-producing β-cells (Liu and Habener, 2010; Welters and Kulkarni, 2008). Evidence supporting this hypothesis includes β-cell hypoplasia following misexpression of the β-catenin inhibitor Axin (Rulifson et al., 2007), and perinatal lethality observed when β-catenin is deleted with an insulin-promoter-driven *Cre* transgene (*RIP-Cre*) (Dabernat et al., 2009). Both of these experiments have the potential for non-specific effects, however. In addition to β-catenin, Axin has been found to inhibit activin–TGF-β signaling through Smad3 (Guo et al., 2008), the latter of which is required for postnatal β-cell expansion (Smart et al., 2006). The *RIP-Cre* transgene, meanwhile, has been shown to induce widespread recombination in the brain (Wicksteed et al., 2010) and to confer metabolic phenotypes independent of deletion in β-cells (Lee et al., 2006). In our hands, *Ngn3-Cre* drives highly efficient deletion of β-catenin in islet cells without apparent phenotype, suggesting that results obtained with *RIP-Cre* must be interpreted cautiously.

Human genetics provides indirect evidence linking Wnt–β-catenin signaling to islet function, because non-coding single-nucleotide polymorphisms (SNPs) in *TCF7L2*, a transcription factor partner of β-catenin, are associated with type-2 diabetes risk (Florez, 2007). Because distinct TCF7L2 isoforms can promote or inhibit Wnt signaling, including within islet cells (Le Bacquer et al., 2011; Tang et al., 2008; Vacik et al., 2011), it is hard to predict how *TCF7L2* polymorphisms might modulate Wnt pathway output, and whether the effects relevant to diabetes are played out in β-cells or in peripheral tissues. Although our results indicate that Wnt–β-catenin signaling is not essential to β-cell function, it will be important to determine whether β-catenin-deficient islet cells adapt normally to metabolic stressors implicated in type-2 diabetes.

Our finding that β-catenin is required differentially in mouse acini and islets emphasizes its cell-type-dependent role in proliferation. Gain-of-function studies similarly indicate that hyperactivation of β-catenin signaling promotes acinar but not islet expansion (Heiser et al., 2006; Strom et al., 2007). Although information on growth control in the human pancreas remains scarce, autopsy-based studies suggest that β-cell and exocrine mass expand with distinct kinetics during childhood (Meier et al., 2008), supporting the hypothesis that proliferation in these compartments is controlled by different mechanisms. Although some aspects of organ growth control might be species specific, our finding that β-catenin is required for all phases of mouse acinar proliferation, from neonatal growth to adult homeostasis and regeneration, suggests that this action of β-catenin is fundamental and ancestral. Studies of rare pancreatic cancer subtypes provide direct evidence for a conserved and acinar-specific role of β-catenin in humans: mutational activation of β-catenin signaling is observed in acinar cell carcinomas but not insulinomas (Abraham et al., 2002), suggesting that the former cancer exploits the proliferation control circuits of its untransformed precursors.

The cell of origin for acinar carcinoma remains unknown, and the activation of β-catenin in this cancer could reflect a role for this pathway in promoting acinar cell identity rather than proliferation per se. Our studies, however, indicate that β-catenin is not required for the phenotypic maintenance of acinar cells, even following injury. During experimental pancreatitis, in particular, we find that β-catenin-deficient and control acinar cells behave similarly in all respects except proliferation. By contrast, Morris et al. found that mice with pan-pancreatic β-catenin deletion, driven by $Ptf1a^{Cre}$, exhibit a more severe response to experimental pancreatitis, including sustained loss of acinar cells (Morris et al., 2010). The difference between this model and ours could reflect the fact that the $Ptf1a^{Cre}$ produces complete β-catenin ablation in acinar cells, whereas a considerable fraction of acinar cells retains β-catenin in ABKO mice. Alternatively, the more severe phenotypes observed with $Ptf1a^{Cre}$ could reflect recombination in non-acinar cells, including ducts (Kawaguchi et al., 2002). Intact duct cell function is required for maintenance of the entire exocrine compartment, as indicated by deletion of the ciliogenesis regulator *Kif3a* (Cano et al., 2006). Although primary cilia are found only on duct cells, their ablation produces a pancreatitis-like phenotype that includes non-cell-autonomous acinar cell death. Interestingly, *Kif3a* knockout mice exhibit fat infiltration into the exocrine pancreas, a phenotype that is frequently seen in severe human pancreatitis (Bockman, 1997) and which can be observed even in uninjured, pan-pancreatic β-catenin knockout mice (Morris et al., 2010; Wells et al., 2007) (L.C.M., unpublished data). We have never observed this phenotype in ABKO mice, suggesting that it reflects a role of β-catenin in multipotent progenitor or mature duct cells, rather than a cell-autonomous function in maintaining acinar survival or differentiation.

The downstream effectors of β-catenin in acinar cells remain undefined, although the Wnt–β-catenin target gene *Myc* is a strong

candidate because its deletion abolishes the exocrine hyperplasia of *Apc* knockout mice (Strom et al., 2007). The apparently reciprocal phenotypes observed upon deletion and activation of β-catenin suggest that it acts as a signaling molecule in the pancreas, although we note that β-catenin can function independently of Wnt ligands (Nelson and Nusse, 2004). Although additional experiments will be required to determine the precise role of Wnt proteins per se in acinar proliferation, small-molecule modulators of β-catenin signaling are increasingly available (Rey and Ellies, 2010) and could be clinically useful. Human pancreatitis, both acute and chronic, is associated with acinar cell proliferation (Ebert et al., 1999; Zimmermann et al., 2002). By enhancing proliferation, pharmacological β-catenin agonists might enhance regeneration and improve the outcome of acute pancreatitis. By contrast, clinical observations indicate that the pain of chronic pancreatitis will occasionally decline in parallel with a decline in acinar cell function, possibly due to the 'burn-out' of residual acinar cells (Sakorafas et al., 2007). By inhibiting regeneration, β-catenin antagonists might accelerate this phenomenon, and perhaps limit overall disease severity. Given that Wnt–β-catenin signaling is active and important in other adult tissues, such as the intestine, effective interventions might require identifying pancreas-specific effectors of this pathway. Nonetheless, the widely used psychiatric agent lithium is now recognized as a potent activator of β-catenin, owing to its inhibition of the GSK3 kinase (O'Brien and Klein, 2009). The fact that patients tolerate long-term lithium treatment suggests that a degree of Wnt–β-catenin modulation can be achieved without prohibitive side effects.

We note that the specific and circumscribed phenotypes of ABKO mice contrast with those of knockouts in other developmental signaling cascades, including Notch and Hedgehog. Inhibiting these pathways, in the context of pancreatitis, causes increased cell death and prevents the resolution of metaplasia (Fendrich et al., 2008; Siveke et al., 2008), implying that small molecules targeting Notch or Hedgehog will have pleiotropic effects, including the alteration of differentiation states. We and others have shown that acinar cells transdifferentiate during *Kras*-induced initiation of pancreatic ductal adenocarcinoma, and that this process is accelerated by pancreatitis (Carriere et al., 2009; De La O et al., 2008; De La O and Murtaugh, 2009; Habbe et al., 2008; Ji et al., 2009; Morris et al., 2010), emphasizing the importance of studying the mechanisms controlling growth and maintenance of this cell type. Understanding the cell-autonomous and non-autonomous roles of β-catenin in acinar cell expansion, injury and regeneration might shed light on the linked etiologies of pancreatitis and pancreatic cancer, as well as identify a molecular pathway that could be harnessed in treating these generally intractable conditions.

METHODS

Mouse breeding and genetic manipulation

All mouse experiments were performed according to a protocol approved by the University of Utah IACUC. The following mice were obtained from the Jackson Laboratories: floxed and germline β-catenin loss-of-function mice (*Ctnnb1*$^{tm2Kem/J}$ and *Ctnnb1*$^{tm2.1Kem}$; referred to here as *Ctnnb1*^{lox} and *Ctnnb1*^{Δ}, respectively) (Brault et al., 2001); the Cre-dependent EYFP reporter strain *Gt(ROSA)26Sor*$^{tm1(EYFP)Cos}$ (Srinivas et al., 2001), referred to here as *R26R*^{EYFP}; and *Ngn3Cre* BAC transgenic mice (Schonhoff et al., 2004). *Pdx1Cre* and *Elastase-CreERT* (*ElaCreERT*) transgenic mice (Gu et al., 2002; Murtaugh et al., 2005; Stanger et al., 2005) were provided by Doug Melton (Harvard University, MA). *Ctnnb1*^{Δ} and *Cre* transgenic lines were maintained by outcrossing to CD-1 wild-type mice, whereas the *Ctnnb1*^{lox} and *R26R*^{EYFP} alleles were maintained by inbreeding on a mixed CD-1 × C57BL/6 background. Unless otherwise noted, experiments were performed in young adult mice, 8-12 weeks in age at the outset of any treatment. To activate recombination by *ElaCreERT*, we administered tamoxifen (Sigma), dissolved in corn oil, by oral gavage.

Glucose tolerance tests

Mice were fasted overnight with access to water and injected with D-glucose at 2 mg/g body weight. Blood was drawn from a tail incision before glucose injection and at indicated time points post-injection, and read with an Ascencia Contour glucometer (Bayer). Net area-under-curve (AUC) values were calculated by the trapezoidal rule. Results are reported as mean ± s.e.m.

Caerulein treatment

We induced acute pancreatitis by caerulein treatment of 2-month-old male and female mice, conditionally null for β-catenin (*Ctnnb1*$^{Δ/lox}$; *R26R*$^{EYFP/+}$; *ElaCreERT*) as well as control littermates (*Ctnnb1*$^{+/lox}$; *R26R*$^{EYFP/+}$; *ElaCreERT*). Following an established protocol (Jensen et al., 2005), mice received repeated intraperitoneal (i.p.) injections of caerulein (Bachem; 0.1 μg/g in 0.8% NaCl) eight times daily over 2 days (16 injections total). Negative controls were injected in parallel with saline alone. We refer to the last day of injections as 'day 0', such that mice sacrificed 48 hours after the last injection would constitute the 2-day post-treatment group. To monitor serum amylase levels, cheek bleeds of approximately 100 μl were drawn, chilled on ice and clarified by centrifugation. Serum samples were diluted 1:4 with PBS, and 7 μl of diluted serum mixed with 280 μl Infinity serum amylase reagent (Thermo-Fisher) prior to analysis on a VMax Kinetic microplate reader (Molecular Devices).

Tissue processing and staining

Mice were euthanized with isoflurane, and tissues were dissected into ice-cold PBS for further processing. BrdU labeling was used to identify proliferating cells, either by injecting mice with BrdU (50 μg/g body weight) at 1 hour prior to sacrifice, or by administering it in the drinking water (1 mg/ml) over 7 days. Pancreata were dissected into multiple fragments and fixed either for paraffin sections [zinc-buffered formalin (Polysciences), overnight] or frozen sections (4% paraformaldehyde/PBS; 4°C 2-4 hours), followed by processing as previously described (Kopinke and Murtaugh, 2010; Murtaugh et al., 2005). Paraffin sections were cut at 6 μm thickness and collected serially such that sections were spaced approximately 50-80 μm apart, spanning the entire specimen. Frozen sections were cut at 8 μm thickness, and similarly collected such that sections were spaced approximately 60-100 μm apart, spanning the entire specimen.

Primary antibodies used for immunostaining are listed in supplementary material Table S1, and secondary antibodies (raised in donkey) were obtained from Jackson ImmunoResearch.

TRANSLATIONAL IMPACT

Clinical issue

Understanding how growth and regeneration of the pancreas are controlled should provide new insights into the etiology and treatment of diseases that affect this organ, including diabetes, pancreatic cancer and pancreatitis. The latter two of these seem to be linked: recent work indicates that pancreatitis can promote acinar cell reprogramming into pancreatic ductal tumors. The Wnt signaling pathway (in which β-catenin is a key mediator) is a crucial regulator of embryonic acinar cell development, but its role in the mature pancreas is more controversial. The numerous proposed roles of β-catenin in both endocrine and exocrine cells of the pancreas are complex and controversial. Some conflicting results have been obtained by manipulating the mouse β-catenin gene with various *Cre* deleter transgenes, most of which are active in multipotent progenitor cells. In this study, the authors sought to strictly define the genetic requirement for β -catenin in the postnatal pancreas, particularly in establishing, maintaining and regenerating its most abundant constituent, the acinar cell.

Results

The authors developed a quantitative 'pulse-chase' knockout and labeling approach to analyze β-catenin function specifically in differentiated acinar cells during their expansion and regeneration. Using this system, they find that β-catenin-deficient acinar cells are dramatically impaired in their ability to proliferate during normal juvenile growth and adult homeostasis, as well as during regeneration from injury caused by experimental pancreatitis. However, β-catenin deletion in acinar cells does not cause cell death or dedifferentiation, nor render the organ more vulnerable to injury. Importantly, the requirement of β-catenin for proliferation seems to be acinar cell specific, because its deletion in islet cells does not detectably impair their expansion or metabolic function.

Implications and future directions

These results clarify the cell-type specificity of β-catenin function in vivo, which can be detected only through a lineage-restricted deletion approach. The Wnt–β-catenin pathway has been implicated in regeneration and tumorigenesis of other tissues, and it is becoming increasingly amenable to pharmacological manipulation as new drugs are developed. For example, small-molecule activators of β-catenin signaling already exist, and this study suggests that they could be useful in accelerating recovery from acute pancreatitis. By contrast, β-catenin inhibitors might prevent the continuous regeneration that sustains chronic pancreatitis. The specificity of β-catenin action implies that such interventions will not affect differentiation state, and therefore should not increase cancer risk. Given that the absence of β-catenin did not disrupt islet cell growth or function, these results also suggest that type-2-diabetes-associated mutations in *TCF7L2* (encoding a β-catenin binding partner) might influence diabetes risk via functions other than in the pancreas. Future studies will focus on the molecular mechanisms upstream and downstream of β-catenin in acinar cells, and address the translational potential of β-catenin-focused intervention in disease models.

Immunohistochemistry and immunostaining were performed as previously described (Kopinke and Murtaugh, 2010; Murtaugh et al., 2005), with all paraffin sections subjected to high-temperature antigen retrieval. To detect Ki67 and BrdU, sections were subjected to digestion with DNAse I (700 u/μl, in 40 mM Tris-HCl pH 7.4, 10 mM NaCl, 6 mM $MgCl_2$, 10 mM $CaCl_2$) for 15 minutes at room temperature (Ye et al., 2007). Slides stained by immunofluorescence were mounted with Fluoromount-G (Southern Biotech) and imaged on an Olympus IX71 microscope. Photomicrographs were produced with MicroSuite software (Olympus) and processed in Adobe Photoshop, with exposure times and adjustments identical between treatment groups.

Image quantification and analysis

To determine $R26R^{EYFP}$ labeling indices, we photographed six to ten independent fields (20× or 40× original magnification) per specimen, across multiple sections. Using ImageJ software (NIH), cells co-expressing a given differentiation marker with EYFP were detected by additive image overlay of the DAPI channel with anti-GFP and anti-marker immunofluorescence, and counted using the Analyze Particles function, as described previously (Kopinke and Murtaugh, 2010). Random samples were scored manually in Adobe Photoshop, to confirm overall counting accuracy. Under staining conditions in which additive image overlay was inaccurate, we scored samples manually in Adobe Photoshop CS5, using the Analysis>Count Tool function. Calculations were performed in Microsoft Excel, and all results are reported as mean ± s.e.m. *P*-values were calculated by two-tailed, unpaired *t*-test.

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COMPETING INTERESTS

The authors declare that they do not have any competing or financial interests.

AUTHOR CONTRIBUTIONS

L.C.M. and M.D.K. conceived and designed the experiments. M.D.K., H.W. and A.K. performed the experiments. J.-P.D.L.O. and M.A.F. contributed input and experimental support to the caerulein treatment experiments. L.C.M. and M.D.K. analyzed the data and wrote the paper.

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SUPPLEMENTARY MATERIAL

Supplementary material for this article is available at
http://dmm.biologists.org/lookup/suppl/doi:10.1242/dmm.007799/-/DC1

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CHAPTER 3

ACINAR CELL PROLIFERATION, NUCLEARITY, AND PLOIDY ARE DIFFERENTIALLY REGULATED DURING POSTNATAL AND REGENERATIVE GROWTH

Abstract

The exocrine pancreas and its mature cell types are maintained by the proliferation of differentiated cells early in postnatal life, as the organ expands together with the organism, but become mostly quiescent in adulthood. We find that the transition towards quiescence in the mouse pancreas is accompanied by an increase in the proportion of binucleate acinar cells, which are present in the human pancreas as well. Further, we demonstrate that mononucleate acinar cells preferentially contribute to juvenile and regenerative growth, indicating the existence of subpopulations within the exocrine pancreas with distinct capacities for growth. Binucleation normally occurs over a narrow postnatal window, and manipulation of acinar proliferation rates, through activation or inhibition of β-catenin, reveals that the decision to undergo binucleation is regulated independent of cell cycle entry itself. Binucleate acinar cells are initially inhibited from further dividing, but regain the ability to divide in the adult organ. Although

overall acinar proliferation in the adult organ is very rare, the division of binucleate acinar cells is sufficient to ultimately generate a significant fraction of tetraploid, mononucleate acinar cells in the adult pancreas, which might themselves contribute to aneuploidy in this cell type. When acinar cells undergo regenerative proliferation in response to injury, however, cell cycle entry is again restricted to mononucleate cells, recapitulating early postnatal expansion and protecting against generation of aneuploid-vulnerable offspring. Our results indicate the existence of novel regulatory programs controlling acinar cell proliferation, the dysregulation of which could produce genome instability and contribute to the capacity of acinar cells to generate pancreatic cancer.

Introduction

Mouse models of pancreatic development have provided important information about the diversity of function between mature cell types derived from a common pancreatic progenitor, but have not identified any proliferative heterogeneity within any single lineage of the mature pancreas [1,2]. The three main cell types of the pancreas: endocrine, acinar, and duct cells, each serve a highly specialized function critical for metabolic processes that sustain life in the organism. Endocrine cells cluster into islets of Langerhans and secrete hormones that regulate blood glucose levels. Acinar cells are dedicated to the synthesis and secretion of digestive enzymes, while duct cells convey these enzymes to the intestine via a highly branched epithelial network. Acinar cells represent the majority cell type in the pancreas and comprise the bulk of

pancreatic mass. Several studies, including our own, have shown that the proliferation of differentiated acinar cells is essential for their expansion during postnatal and regenerative growth [3-5]. What is not known is whether all acinar cells contribute equally to growth of the pancreas, or whether there exist functionally distinct subsets of cells within this lineage that are specifically competent to proliferate. Descriptive studies in the rat indicate the presence of binucleate acinar cells (2x2n DNA content) in the mature pancreas that are refractory to cell cycle entry, potentially implicating nuclearity as a determinant of proliferation potential [6,7]. The existence of binucleate acinar cells introduces the possibility that there may exist a functional heterogeneity within the acinar cell lineage.

Though mononucleate and diploid cells are widely considered the default for vertebrates, a major role for binucleation and diploid-to-polyploid conversion has been described for normal development of cells within the liver, blood, and heart. The biological advantages of polyploidy have been proposed to include an improved response to metabolic demands and buffering against deleterious mutations that may precipitate tumorigenesis[8-10]. There are various mechanisms by which a cell could become binucleate and subsequently polyploid, including cell-cell fusion as occurs in skeletal muscle and osteoclasts, or by incomplete cytokinesis as is seen in cardiomyocytes, trophoblast giant cells, and hepatocytes [8,10,11]. Polyploidization of hepatocytes, which affects >90% of rodent liver cells, begins with incomplete cytokinesis to generate binucleated cells [10]. As the liver shares a common developmental origin with the pancreas, incomplete

cytokinesis represents a likely candidate for the generation of binucleate acinar cells. Interestingly, when a binucleated hepatocyte (2 x 2n) proceeds through another complete cell cycle, it produces two mononucleated tetraploid daughter cells (1 x 4n). Tetraploid cells face the problem of having acquired an extra set of centrosomes that could potentially compromise the assembly of a bipolar spindle, making them prone to aneuploidy if they divide again. In fact, recent studies have determined that over half of mature hepatocytes in mice and humans have an aneuploid chromosome number, and yet they retain the ability to undergo mitosis [12,13]. Whether aneuploid hepatocytes have a higher propensity for tumorigenesis is not known.

In this study, we demonstrate that pancreatic acinar cell nuclearity, proliferation, and ploidy are differentially regulated during juvenile growth and adult regeneration, highlighting a previously unappreciated heterogeneity within this cell type. Furthermore, we identify for the first time binucleate acinar cells in the human pancreas. In addition to defining a new developmentally regulated process in the postnatal pancreas, our studies highlight the potential vulnerability of acinar cells to pathological alterations of chromosome number, which may underlie their ability to give rise to pancreatic cancer.

Results

Binucleate acinar cells are present in mouse and human pancreas

In the mouse and human adult pancreata, the trained eye can readily pick out individual binucleate acinar cells in H&E-stained paraffin sections (Figure 3-1, A and B). These binucleate acinar cells are typified by two nuclei that reside closely together on the basal surface of the cell. Binucleate cells were easily recognized by immunofluorescent staining for E-cadherin, which marks the lateral and apical surfaces separating individual acinar cells (Figure 3-1, C and D). Identifying binucleate cells in section, on this basis, we found that 27.4±1.2% of all acinar cells were binucleate in adult (6-month) mouse pancreata, and 12.7±1.5% in adult human pancreata (Figure 3-1E). We suspected, however, that analysis of nuclearity in sections might systematically underestimate the frequency of binucleate acinar cells, as we excluded ambiguous cells and would necessarily miss cells in which two nuclei had been separated by the plane of section. To be more rigorous and comprehensive, we developed an assay in which we digested the pancreas to a single cell suspension, which was stained on slides as a cytospin preparation. Using antibodies for the acinar cell-specific digestive enzyme amylase and the DNA dye DAPI, we could unambiguously score acinar cells as mono- or binucleate (Figure 3-1, F and G).

Binucleate acinar cells in adult mice were ~1/3 larger (Figure 3-2) than mononucleate acinar cells by surface area, a feature that has also been described for hepatocytes, in which cell size scales with DNA content [10]. We

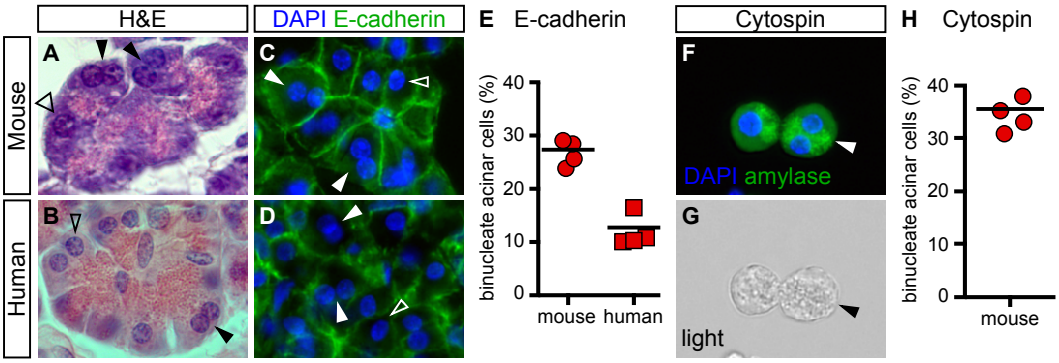

Figure 3-1. Binucleate pancreatic acinar cells are present in mouse and human pancreas. (A-B) H&E staining of adult mouse and human pancreata reveals morphologically distinct mononucleate (open arrowheads) and binucleate (closed arrowheads) acinar cells. (C-D) E-cadherin immunofluorescence (green) on sectioned tissue highlights cell boundaries and identifies individual mouse and human acinar cells containing two DAPI+ nuclei (blue). (E) Quantification of binucleate acinar cell frequency in mouse and human (values (red dot) represent the fraction of binucleate acinar cells per individual mouse or human, n=4 each), based on E-cadherin/DAPI staining. (F-G) Amylase immunofluorescence (green) on cytospin-prepared samples from whole pancreas digestion of adult (P238) mouse reveals mono- and binucleate acinar cells. (H) Quantification of binucleate acinar cell frequency in adult mice (n=4), based on staining of cytospin preparations.

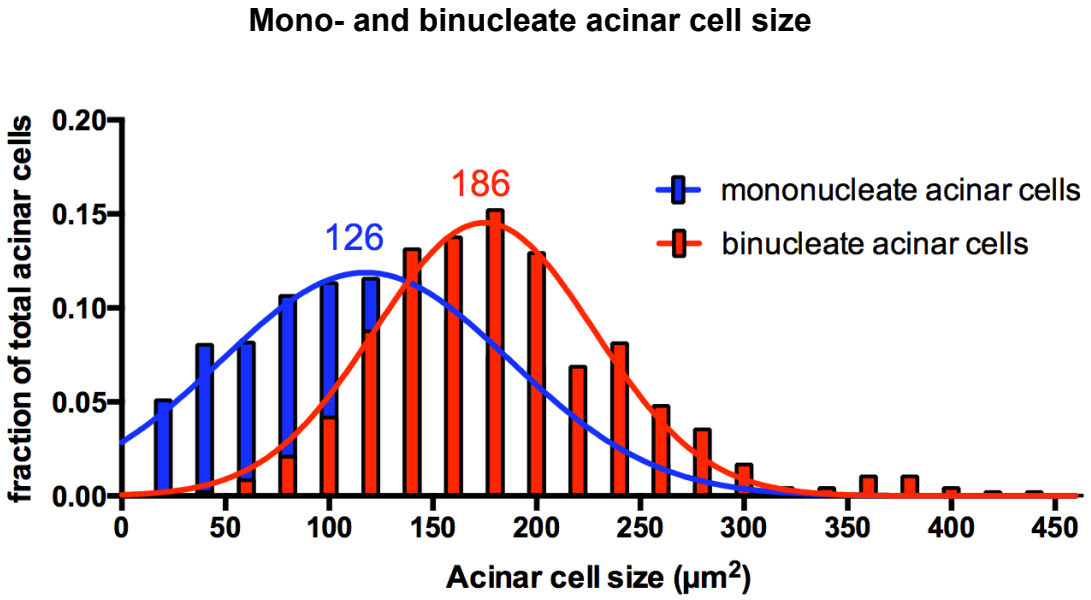

Figure 3-2. Acinar cell size increases with increasing DNA content. Graphing the distribution of mono-and binucleate acinar cell area (μm2) reveals a bimodal distribution. The heights of the blue bars represent the fraction of total acinar cells that are mononucleate, for each bin on the x-axis representing cell surface area. The red bars represent the fraction of total binucleate acinar cells for each bin. A color matched Gaussian curve and has been fit to each data group to illustrate that binucleate acinar cells (mean cell surface area 186 μm2) are ~1/3 larger than mononucleate cells (mean cell surface area 126 μm2) (ANOVA P<0.0001).

found that binucleate acinar cells in the adult pancreas were similar to mononucleate cells morphologically and maintained expression of markers of differentiated acinar cells, contrasting with a previous report that described binucleate acinar cells, generated *in vitro,* as subject to a high frequency of transdifferentiation [14].

Using the dissociation and cytospin technique, we found that 35.4±1.3% of all acinar cells in the adult (6-month) mouse pancreas were binucleate (Figure 3-1H). As these same samples had been analyzed by both section immunofluorescence and cytospin, we could conclude that analysis of sections results in ~25% undercount of binucleate cells compared to cytospin, the latter of which we consider a more definitive method for quantifying acinar cell nuclearity. We hypothesize that our analysis of human acinar cells nuclearity, based as it is on section immunofluorescence, is also likely to undercount the true prevalence of binucleate cells.

Postnatal acinar cell expansion is driven by preferential proliferation of mononucleate cells

Acinar cell numbers expand rapidly in early postnatal life, as the organ grows in size along with the organism. To determine the kinetics of acinar cell binucleation in the mouse pancreas, we performed a timecourse in wild-type CD-1 mice from birth (P0) until 2 years (P730) of age (Figure 3-3A). Prior to weaning (P21), there were very few binucleate acinar cells (<5%). During the suckling-to-weaning transition (~P17), the proportion of binucleate acinar cells increased and,

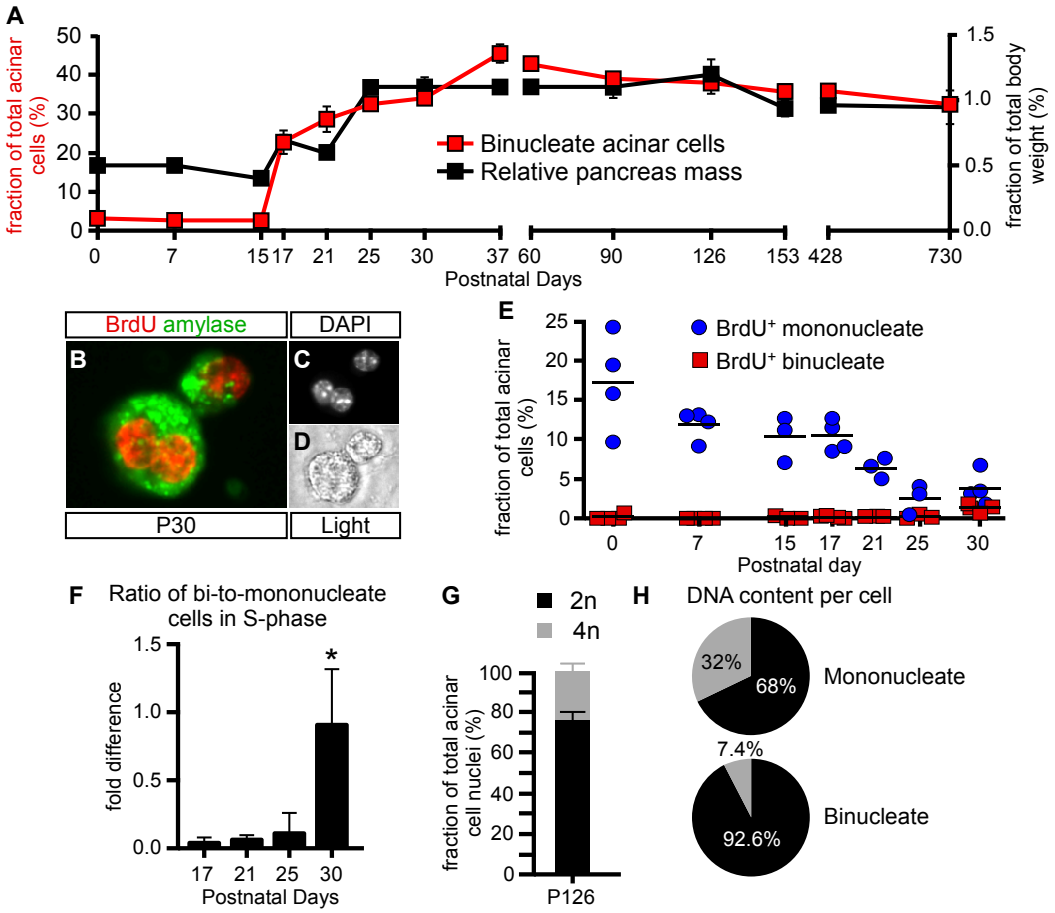

Figure 3-3. Regulated changes in DNA content during postnatal acinar cell maturation. (A) Timecourse of binucleate acinar cell frequency (red) and relative pancreas mass (black) in CD-1 wild-type mice from birth (P0) to 2 years (P730) of age (n=3-9 mice per timepoint). (B-D) Immunofluorescence detection of BrdU+ nuclei (red) in amylase+ acinar cells (green), either mono- or binucleate. (E) Quantification of total acinar cells that are mononucleate BrdU positive (blue) and binucleate BrdU positive (red) during postnatal growth. (F) Relative proportion of binucleate to mononucleate BrdU+ cells, normalizing for the total number of mono- and binucleate cells present at each stage. (G) Proportions of 2n and 4n nuclei in the adult mouse pancreas (P126) (n=3 mice). (H-I) Distribution of mononucleate and tetraploid nuclei within mono- and binucleate acinar cells at this stage. Error bars indicate s.e.m.

by 1 month of age, represented >35% of all acinar cells in the mature pancreas. Intriguingly, this transition coincided closely with an increase in the relative mass of the pancreas between suckling and weaning stages (Figure 3-3A), suggesting that both phenomena may be controlled by signals responding to changing metabolic conditions. From 1 month of age onwards, the relative abundance of binucleate acinar cells remained essentially constant, as did relative pancreas mass (Figure 3-3A), suggesting the possible existence of a homeostatic mechanism regulating acinar cell nuclearity.

Staining cytospin preparations for the acinar cell digestive enzyme amylase and the S-phase marker BrdU allowed us to determine the proportion of mono- and binucleate acinar cells entering S-phase (Figure 3-3, B-D). The overall frequency of acinar cells in S-phase declined throughout the postnatal period (Figure 3-3E). Over the first 3-4 weeks of life, virtually no binucleate acinar cells were observed as being in S-phase, suggesting that increased cellularity is driven by proliferation of only mononucleate acinar cells. Interestingly, in the mature pancreas (P30), the frequency of binucleate acinar cells in S-phase strikingly increased, despite an overall low frequency of acinar cells proliferating (Figure 3-3E). Normalizing for the overall frequency of binucleate acinar cells, binucleate cell proliferation appeared to be actively suppressed during early life. This inhibition relaxes by P30 and binucleate acinar cells undergo DNA synthesis with the same frequency as mononucleate acinar cells (Figure 3-3F). To our knowledge, this is the first *in vivo* demonstration that mammalian cell proliferation can be differentially regulated according to nuclear content.

Regulated changes in DNA content during postnatal acinar cell maturation

Previous studies in the liver have shown that hepatocyte ploidy increases via progression through binucleate intermediates (2 x 2n), which divide to generate two tetraploid, mononucleate daughter cells (1 x 4n) [11,15]. We would predict a similar phenomenon to occur in the pancreas upon cell cycle re-entry of binucleate acinar cells; this could account for previous observations, based on FACS of isolated nuclei, that as many as 40% of mouse acinar cell nuclei contain 4n DNA content [16]. We revisited this issue in our cytospin-based analyses, which permit us to determine whether tetraploidy occurs within mononucleate (1 x 4n) or binucleate cells (2 x 4n).

We developed an assay to quantify nuclear fluorescence of DAPI, which binds stochiometrically to DNA, in stained cytospin preparations, and to determine relative staining intensity among cells of a single mouse and between mice. Nuclear fluorescence was corrected for size and background, and normalized within each sample to the median nuclear intensity of that sample, which we presume to be diploid, to account for experimental variation between mice in our staining procedures. This produced, for each sample, a series of nuclear fluorescence measurements that we referred to as the median corrected nuclear fluorescence, or MNCF. Once we determined the MNCF values for each mouse, we performed a *K*-means cluster analysis to group these values into ploidy peaks and determine, in an unbiased way, the number of peaks capturing the distribution of nuclear DNA content and the proportion of nuclei contained

within each peak.

Using this technique, we found that in the adult pancreas (4 months), 24±4.1% of all nuclei were tetraploid the remainder diploid, with no detectible population containing >4n DNA content (Figure 3-3G). This is consistent with previous studies indicating that the mouse pancreas has a high proportion of tetraploid nuclei and a low proportion of octaploid nuclei [16]. Mononucleate acinar cells, which comprise the majority of cells in the adult pancreas, were 32±7.2% tetraploid (1 x 4n), compared to binucleate cells where only 7.2±1.3% of nuclei were tetraploid (2 x 4n) (Figure 3-3H). The large number of mononucleate tetraploid cells suggests that, similar to hepatocytes, acinar cell ploidy increases through the division of binucleate diploid (2 x 2n) cells to generate mononucleate tetraploid cells (1 x 4n) [11]. These processes impose considerable genomic heterogeneity on adult pancreatic acinar cells, which we now appreciate to vary in both nuclear content per cell and DNA content per nucleus.

Binucleate acinar cells arise via incomplete mitosis

Numerous mammalian cell types undergo multinucleation and/or polyploidization as part of their normal development through mechanisms that include endocycling, cell-cell fusion and incomplete mitosis (i.e., karyokinesis without cytokinesis). Binucleation is widespread among hepatocytes in both mouse and human, and appears to arise via abortive cytokinesis [8,10]. Cultured *in vitro*, acinar cells have been found to undergo abortive cytokinesis to generate binucleate daughter cells, although in this case, the process coincided with loss

of normal acinar differentiation characteristics [14]. To address the origins of binucleate acinar cells, we employed several labeling techniques *in vivo*.

The rapid expansion of binucleate acinar cells at weaning provided a logical time window in which to study the genesis of binuclearity. We reasoned that if binucleate cells arose via cell-cell fusion, the fusion partners might differ in their recent proliferation history, indicated by incorporation of BrdU. By contrast, the two nuclei of a binucleate cell derived by incomplete mitosis would, by definition, have an identical proliferation history. We administered BrdU to CD-1 wild-type mice by daily i.p. injection (50 μg/g) for 6 consecutive days after weaning; after a further 24 hours, to permit cells in S-phase to complete mitosis, mice were euthanized and pancreata analyzed by cytospin to characterize the distribution of BrdU among mono- and binucleate acinar cells. The presence of a single BrdU-labeled nucleus within a binuclear acinar cell would demonstrate an origin from fusion of two separate cells, while two labeled nuclei would be consistent with genesis via abortive mitosis.

We examined >5000 acinar cell nuclei from independent mice, and identified ~1000 BrdU+ mononucleate cells. These were most likely generated via division of mononucleate diploid cells (1 x 2n), although they could also have been produced via cell-cycle re-entry of binucleate acinar cells. Importantly, among all binucleate cells found to have incorporated BrdU during the labeling period (285 cells), we consistently observed two labeled nuclei per cell and never found a binucleate cell with a single BrdU+ nucleus (Figure 3-4A). This finding supports a model of binucleation via incomplete mitosis, although it leaves open

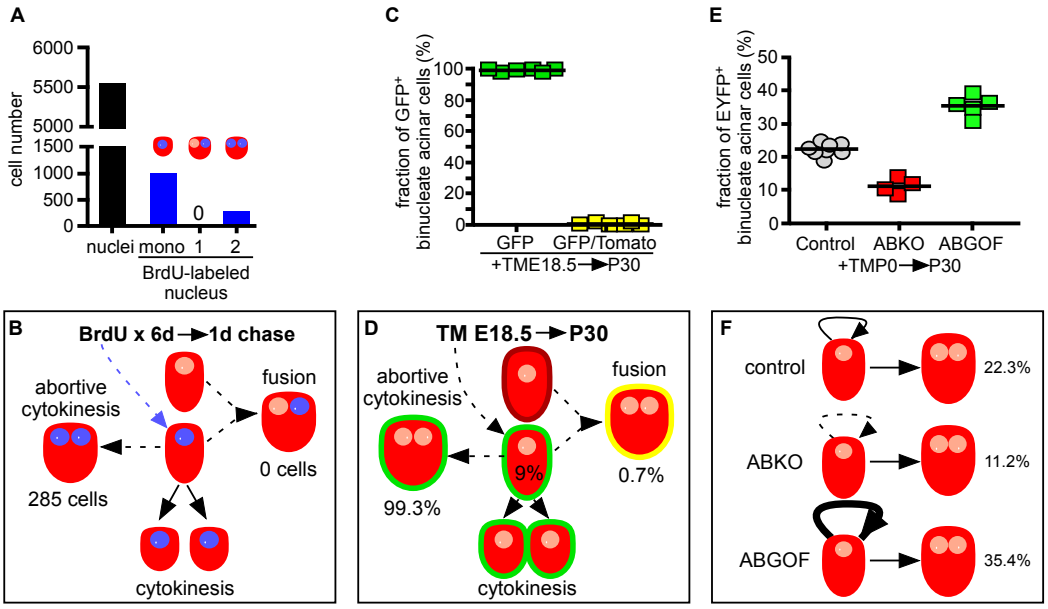

Figure 3-4. Binucleate acinar cells arise via abortive mitosis. (A) Frequency of BrdU incorporation into one vs. two nuclei of binucleate acinar cells, analyzed after multiple BrdU injections over 6 days followed by a 1 day chase period. In total, 285 binucleate cells were observed with BrdU labeling of both nuclei (21±3.7% of all acinar cells), and zero with only one labeled nucleus (n=3 mice). (B) Interpretation of data in (A), indicating that no binucleate cells were found that could have arisen by fusion. (C) Proportion of GFP+ acinar cells in R26RmTmG; Ptf1aCreERT mice, with perinatal tamoxifen administration, expressing GFP alone (green) or co-expressing Tomato (yellow) (>150 binucleate cells scored/mouse, n=6 mice). (D) Interpretation of data in (C), indicating that of the 9% of acinar cells marked by recombination, only a very small proportion could have contributed to binucleate cells via cell-cell fusion as opposed to incomplete mitosis. (E) At P30, when binucleate cells are normally present, we find that ABKO acinar cells exhibit reduced binuclearity compared to controls (11.2±1.8% vs. 22.3±0.7%, respectively, P<0.0001), while ABGOF cells exhibit increased binuclearity (35.4±1.4%, P<0.0001) (n=4-7 mice per genotype). (F) A schematic showing that acinar cells are prone to undergo binucleation as a function of their overall cell division rate, whereby increasing or decreasing the number of cells proceeding through the cell cycle correspondingly changes the number of cells susceptible to incomplete mitosis during a critical weaning period.

the formal possibility that binucleate acinar cells are generated by the selective fusion of two acinar cells that have both, independently, gone through a recent cell cycle (i.e., both BrdU+).

As an alternative approach to detect cell-cell fusion during binucleation, we used the double-fluorescent *R26R*^{mTmG} reporter mouse in which cells switch expression from red to green fluorescent proteins (membrane-tagged Tomato and EGFP, respectively) depending on whether the reporter has undergone Cre-mediated recombination [17]. We sought to establish conditions in which a minority of acinar cells would undergo recombination prior to binucleation, i.e., a small number of green cells within a red background. The expression of green and red fluorescent proteins should remain mutually exclusive unless cell-cell fusion occurs, in which an un-recombined cell (red) and a recombined cell (green) would fuse to produce a binucleate "yellow" cell. We used the *Ptf1a*^{CreERT} deletor line for this purpose, in which administration of tamoxifen during late fetal life results in mosaic, acinar-specific recombination [18]. Pregnant females from a cross between the *R26R*^{mTmG} reporter and *Ptf1a*^{CreERT} were administered tamoxifen at embryonic day 18.5 (E18.5), and their double-transgenic (*Ptf1a*^{CreERT}*; R26R*^{mTmG}) offspring (n=4) sacrificed at 1 month of age for pancreatic cytospin analysis.

The dose of tamoxifen used (5 mg/30 g mouse) resulted in EGFP labeling of 9±1% of all acinar cells (Figure 3-4D). Among the EGFP+ binucleate acinar cells (>150 binucleate cells examined per mouse, n=6 mice), we found that 99.3±0.4% were Tomato-negative, consistent with an origin via abortive mitosis

of a mononucleate EGFP+ labeled cell (Figure 3-4, C and D). The tiny fraction of EGFP+/mTomato+ cells observed (0.7±0.4%, 5 total cells in n=6 mice) could indicate very rare fusion events, or else represent mosaic labeling of rare binucleate cells already present in late fetal/neonatal life. Given that mTomato+ cells represent the majority of "partner" cells available for fusion, the overwhelming predominance of EGFP-only labeled binucleate acinar cells strongly argues against an origin via fusion and in favor of incomplete mitosis.

Acinar cell nuclearity is regulated independent of the decision to enter the cell cycle

We have previously shown, via conditional knockout approaches, that the multifunctional protein β-catenin is required for proliferation of acinar cells during postnatal and regenerative growth of the pancreas [3]. β-catenin plays key roles in both Wnt signaling and cell-cell adhesion, the former of which are regulated by inhibitory phosphorylation sites near the N-terminus of the protein. Removing these sites, via deletion of exon 3 of the β-catenin gene (*Ctnnb1*) in which these residues are encoded, produces a stabilized and constitutively active protein [19]. Consistent with previous studies [4], we found that acinar-specific β-catenin gain-of-function (ABGOF), via deletion of *Ctnnb1* exon 3 in *Elastase-CreERT* deletor mice [20], accelerates acinar cell proliferation throughout postnatal development and therefore increases pancreatic mass in the adult (Figure 3-5, A-C) The fact that ABGOF produces a reciprocal phenotype to our previously reported acinar-specific β-catenin knockout (ABKO) not only supports a key role for Wnt signaling

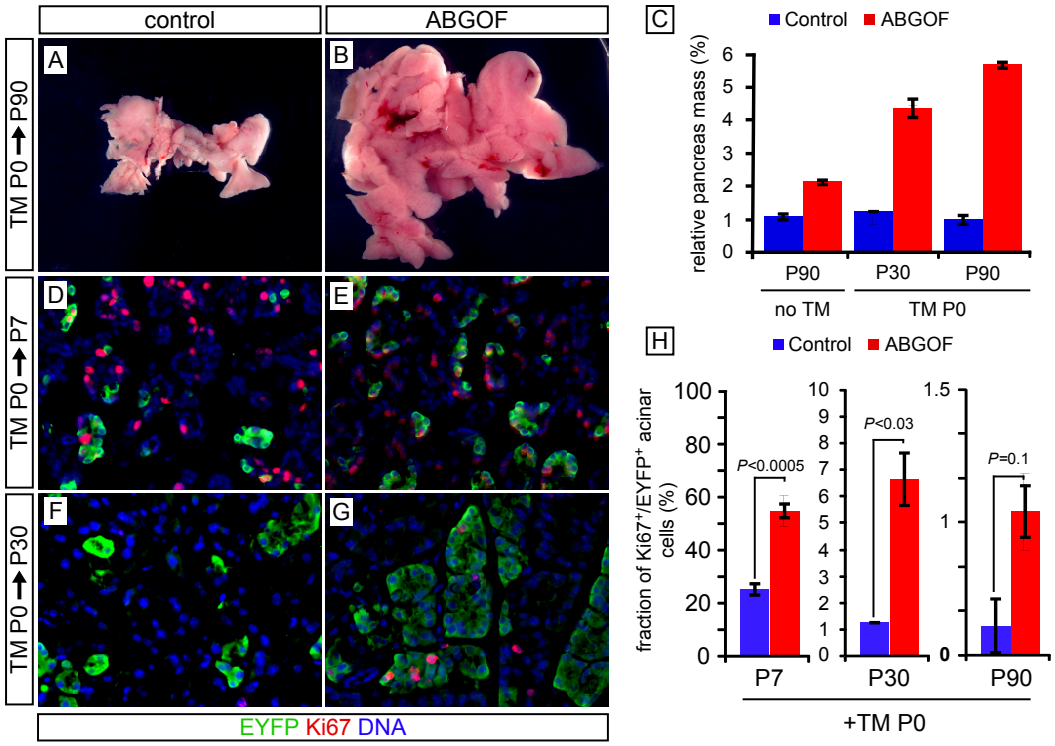

Figure 3-5. β-catenin is sufficient to increase proliferation and pancreatic mass during postnatal growth. Control or ABGOF mice received tamoxifen (TM) either on the day of birth, via maternal gavage, or post-weaning (P21, P30, P60) (n=4-8 mice per genotype per timepoint). (A, B) At P90, pancreata of ABGOF mice that received TM at birth are visibly larger than those of control littermates (photographs taken at identical magnification). (C) The relative pancreas mass was determined for P90 control and ABGOF mice that received TM at several postnatal timepoints and compared to mice that received no TM. ABGOF mice that received TM at birth had pancreata that were 5-fold larger than littermate controls. However, when TM was administered at P21, P30, and P60, the stabilization of β-catenin was not sufficient to increase pancreas mass compared to no-TM controls. (D-G) Staining for EYFP lineage labeling (green) acinar cells and the proliferation marker Ki67 (red) reveals relatively more EYFP+Ki67+ cells in the ABGOF pancreata at P7 (D, E) and P30 (E, F), compared with controls. Scale bar: 100μm. (H) Quantification of Ki67 labeling index of EYFP+ acinar cells reveals a significant increase in the proliferative capacity of acini harboring a stabilized allele of β-catenin compared with control mice that have only the wild-type β-catenin allele.

in acinar proliferation, but also provides a tool to determine the impact of proliferation rate on acinar cell nuclearity.

The genetic crosses used to generate β-catenin loss- and gain-of-function mice are depicted in Figure 3-6. Importantly, both the ABKO and ABGOF models (as well as controls expressing *ElaCreERT* alone) were established with a $R26R^{EYFP}$ reporter construct in the background, permitting tamoxifen-inducible EYFP labeling of recombined acinar cells [3,21]. By comparing the nuclearity of EYFP-labeled acinar cells among identically treated control, ABKO, and ABGOF mice, we should detect the effect that proliferation rate per se has on binucleation during postnatal development. To conditionally delete or activate β-catenin in mice, we administered 10 mg tamoxifen to nursing females at birth (P0) and analyzed EYFP labeling after a "chase" period of 7 or 30 days. As previously described [3], we found widespread EYFP expression by acinar cells of control and ABKO mice and comparable EYFP expression in ABGOF mice. At P30, when binucleate cells are normally present, we find that ABKO acinar cells exhibit reduced binuclearity compared to controls (11.2±1.8% vs. 22.3±0.7%, respectively), while ABGOF cells exhibit increased binuclearity (35.4±1.4%) (Figure 3-4, E and F). This result suggests that acinar cells are prone to undergo binucleation as a function of their overall cell division rate, such that increasing or decreasing the number of cells proceeding through the cell cycle correspondingly changes the number of cells susceptible to incomplete mitosis during the critical weaning period.

Alternatively, β-catenin signaling itself might directly regulate acinar cell

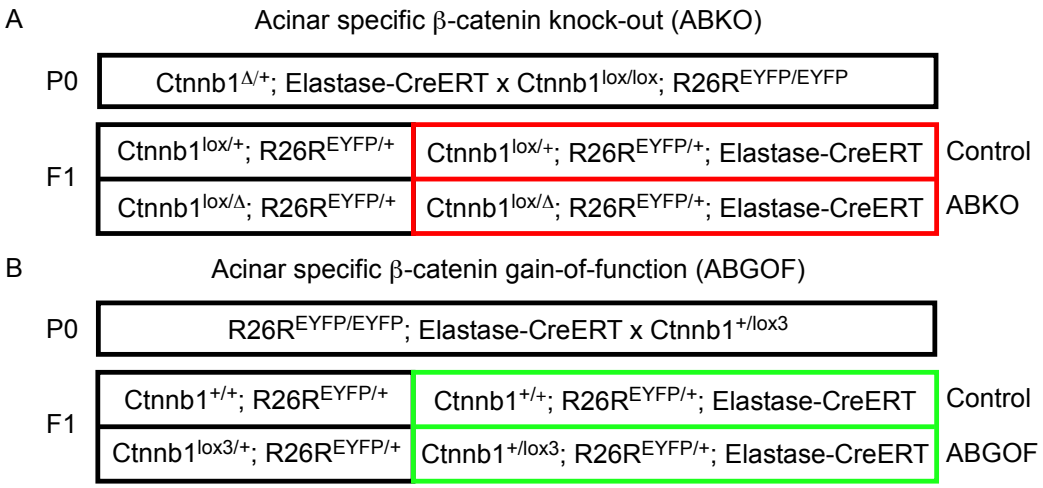

Figure 3-6. Breeding for ABKO, ABGOF mice and their sibling controls.

nuclearity via, for example, postulated effects of this protein on mitotic spindle function [22]. To test this, we examined control, ABKO, and ABGOF mice at P7, when binucleate acinar cells are rare but overall proliferation rates are high. As expected, proliferation of ABKO acinar cells was reduced relative to control at P7 as previously shown [3], while that of ABGOF cells was increased (Figure 3-5, D-H). By contrast, there was no difference at this age in the fraction of mono- and binucleate acinar cells between control, ABKO, or ABGOF (data not shown). Taken together, these results suggest that β-catenin regulates acinar cell nuclearity indirectly, by controlling overall proliferation rate during the critical weaning period in which binucleate cells are first established. Our findings further highlight the uniqueness of this transition: while proliferation itself is high at P7, nearly all cells complete mitosis and generate mononucleate offspring; just over a week later, overall proliferation rates have declined, yet a substantial fraction of cell cycles become diverted to incomplete mitosis and binucleation.

The selective division of mononucleate acinar cells drives acinar cell regeneration

Previous studies in the rat have indicated that only mononucleate acinar cells are capable of proliferation throughout postnatal life, such that the generation of binucleate cells represents a terminal differentiation step [6,23]. By contrast, we have shown here that binucleate cells of the mature mouse pancreas can re-enter the cell cycle to generate tetraploid offspring (Figure 3-3). Although the overall low rate of cell division in the resting adult pancreas is

predicted to limit the generation of tetraploid cells, acinar cell proliferation is known to increase dramatically in experimental models of acute pancreatitis, in which surviving acinar cells repopulate those lost to injury [3,5,24]. If binucleate cells were driven to re-enter the cell cycle after injury, they would be predicted to generate tetraploid daughter cells and, subsequently, aneuploid "granddaughters" [8]. Such a link between injury and genome instability could account for the well-established contribution of pancreatitis to pancreatic cancer risk.

To determine regenerative proliferation involves both mono- and bi-nucleate acinar cells; we induced acute pancreatitis by supramaximal caerulein stimulation, as previously described [3,25]. Monitoring serum amylase levels before and after caerulein administration confirmed transient elevation, diagnostic of pancreatic injury (data not shown). Pancreata were analyzed by cytospin at various timepoints post-injury, including during the period of peak injury-induced proliferation (2 d) and after regeneration and histological normalization (≥7 d). All animals received BrdU 1 hour prior to euthanasia, to label cells in S-phase. Saline-injected control pancreata exhibited the usual distribution of mononucleate acinar cells [2 d Saline (n=7); mononucleate 67.8±2.4%], while the pancreata of caerulein-treated mice exhibited a significant increase in the fraction of mononucleate cells 2-days post-caerulein treatment [2 d Caerulein (n=7); mononucleate 82.1±1.5% $P<0.002$] (Figure 3-7A). Although this increase is transient, as the normal distribution of mono- and binucleate cells is restored by 7 d post-injury, it suggests that the genesis and division of mono- and binucleate cells might be regulated differentially during regeneration. Indeed, BrdU staining

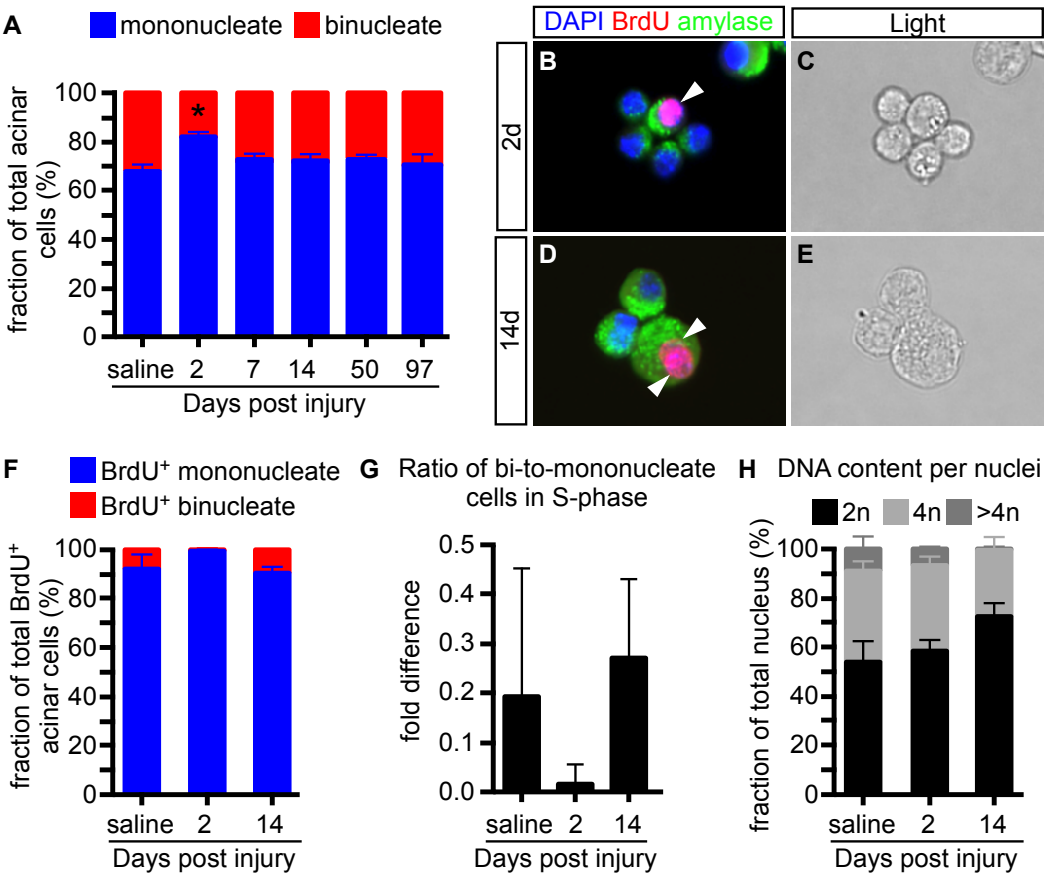

Figure 3-7. Acinar cell regeneration is driven by the selective division of mononucleate acinar cells. (A) Proportions of mono- and binucleate acinar cells in pancreata of uninjured (saline-treated) mice vs. mice sacrificed with increasing time after caerulein-induced pancreatic injury (n=6 mice per genotype per treatment group). Increased mononucleate cells are observed specifically at 2 days post-injury (P<0.002). (B-E) Immunofluorescence and brightfield images of acinar cells from mice at 2- or 14-days post-caerulein treatment, stained for BrdU (red), amylase (green), and DAPI (blue). (F) Distribution of BrdU labeling between mono- and binucleate acinar cells of uninjured (saline) or caerulein-treated mice. (G) Relative proportion of mononucleate to binucleate BrdU+ cells in control and injured pancreata, normalizing for the total number of mono- and binucleate cells present in each sample. (H) Analysis of acinar cell ploidy, by quantification of DAPI fluorescence, in control and injured pancreata. Error bars indicate s.e.m.

revealed that binucleate acinar cells were selectively inhibited from proliferation during regenerative proliferation, relative to their proliferative capacity before and after injury (Figure 3-7, B-G). Thus, similar to juvenile/weaning stages (Figure 3-3F), our results indicate the existence of a mechanism to selectively restrict cell cycle entry of binucleate cells during the period of rapid growth that occurs after injury.

Given that binucleate cells (2 x 2n) spontaneously generate tetraploid, mononucleate daughter cells (1 x 4n), we reasoned that examination of DNA content per nucleus could provide independent insight into the ability of binucleate acinar cells to divide after injury. Injury of the liver drives binucleate hepatocytes (2 x 2n) to divide and produce a net increase in ploidy through generation of tetraploid and octaploid mononucleate hepatocytes [11,26]. By contrast, we found that the frequency of tetraploid nuclei either remained constant or slightly decreased after injury, consistent with a selective lack of division of binucleate cells (Figure 3-7H). As a result of this novel cell cycle control mechanism, the acinar compartment is prevented from accumulating additional polyploid cells during regenerative proliferation, which could otherwise contribute to risk of aneuploidy and chromosome instability.

Discussion

During postnatal and regenerative growth of the pancreas, new acinar cells are generated almost exclusively from the division of pre-existing acinar cells, but no one has established whether all acinar cells have the same capacity

for growth or whether there exists a distinct subpopulation of cells with this capacity [1,3,5,24,25,27]. We found that postnatal growth in the mouse pancreas is accompanied by an increase in the proportion of binucleate acinar cells after weaning, which are also present in the adult human pancreas. We have additionally defined a proliferative heterogeneity within the acinar cell lineage, in which mononucleate acinar cells preferentially contribute to juvenile and regenerative growth, identifying the existence of subpopulations within the exocrine pancreas with distinct capacities for growth. To our knowledge, this is the first study to define the capacity of mononucleate acinar cells for postnatal and regenerative growth in mice, as well as identify their existence in the human pancreas.

Pancreatic regeneration following caerulein-induced acute pancreatitis is the result of a highly organized process of tissue remodeling. The focus of this study has been to define the cellular origin for this process, which previous studies have determined proceeds by the expansion of surviving acinar cells [1,3,5,24,25,27]. What has not been shown is whether all acinar cells have the same capacity for regenerative growth and therefore contribute equally to expansion of the acinar cell compartment after injury. We find that the burst of regenerative proliferation driven by caerulein treatment is confined to mononucleate acinar cells, whereas proliferation before and after injury occurs at similar levels between mono- and binucleate cells. The restricted proliferation of mononucleate acinar cells parallels the situation in the juvenile pancreas, potentially indicating another instance of regeneration recapitulating development. One net result of

limiting the proliferation of binucleate cells is to limit the generation of tetraploid daughter cells, which have duplicated centrosomes and frequently produce aneuploid offspring [8]. This has been directly observed in the liver, where regenerative proliferation causes widespread aneuploidy among hepatocytes [28]. Tissue culture studies of mammalian cells have previously indicated that binucleate cells are not inhibited from cell cycle-progression [8,29]. Our finding that such an inhibition is imposed during postnatal and regenerative growth implies the existence of a novel mechanism of cell cycle control in acinar cells.

While the cause-and-effect relationship of aneuploidy to carcinogenesis is controversial, pancreatic ductal adenocarcinoma frequently exhibits triploid or greater chromosome number, beginning from its precancerous lesions [8,30,31]. As this cancer can arise from acinar cells [32,33], the existence of binucleation among human acini could establish a population vulnerable to chromosome gain and loss during cancer formation. Pancreatic cancer is known to depend on activation of the oncogene *KRAS* and loss of tumor suppressor genes such as *P53*, manipulations of which have been exploited to model this disease in mice [34]. To determine if mutations in these genes themselves alter pancreatic nuclearity or ploidy, we examined *Kras$^{LSL\text{-}G12D}$; Pdx1-Cre* (pancreas-specific *Kras* activation), and *p53$^{lox/lox}$; Pdx1-Cre* (pancreas-specific *p53* knockout) mice, but did not observe changes in either the frequency of binucleate or tetraploid cells at two months of age (data not shown). While alterations of *KRAS* and *P53* may not directly affect acinar DNA content, the susceptibility of tetraploid acinar cells to chromosome gain and loss could contribute to copy-number changes, including

loss-of-heterozygosity (LOH), that frequently affect these and other genes in pancreatic cancer.

The genomic heterogeneity of acinar cells has practical implications for mouse genetic studies: insofar as subpopulations of cells carry extra copies of loxP-flanked conditional knockout alleles, for example, they may be less prone to complete deletion by Cre recombinase. On the other hand, if binucleate and tetraploid cells give rise to chromosomally-unstable offspring, they may preferentially contribute to cancer in models that require tumor suppressor gene LOH, or where malignancy is driven by aneuploidy. Devising a method to specifically mark and trace binucleate or tetraploid cells may be necessary to determine their physiological and pathological roles. Of equal importance are the mechanisms by which these cells arise, and by which their genesis is regulated during growth and regeneration. Regarding the former, recent studies in liver have implicated insulin signaling in hepatocyte binucleation [35], and it is plausible that this pathway could act on pancreatic acinar cells as well. Understanding how binucleated cells might be selectively prevented from re-entering the cell cycle is more of a challenge, given that a similar checkpoint has not been demonstrated in other cells. Nonetheless, inroads into this mechanism could permit therapeutic interventions to selectively enhance the proliferation of mononucleate acinar cells, promoting exocrine growth and regeneration without increasing a population of aneuploid-prone cells with a propensity to generate cancer.

Methods

Mouse breeding and treatment

All mouse experiments were performed according to protocols approved by the University of Utah Institutional Animal Care and Use Committee. Floxed β-catenin loss-of-function mice (*Ctnnb1*$^{tm2kem/J}$, referred to here as *Ctnnb1*^{lox}) [36] and the Cre-dependent EYFP reporter strains *Gt(ROSA)26Sor*$^{tm1(EYFP)Cos}$ (referred to here as *R26R*^{EYFP}) [21] and *Gt(ROSA)26Sor*$^{tm4(ACTB-tdTomato,-EGFP)Luo}$ (referred to here as *R26R*^{mTmG}) [17] were obtained from the Jackson Laboratory (Bar Harbor, ME). Conditional stabilized β-catenin mice (*Ctnnb1*^{tm1Mmt}*,* referred to here as *Ctnnb1*^{lox3}) [19]were obtained from Makoto Mark Taketo (Kyoto University, Kyoto, Japan). *Ptf1a*$^{CreERT/+}$ knock-in [18] and *Elastase-CreERT (ElaCreERT)* transgenic mice [20] were provided by Chris Wright (Vanderbilt University, Nashville, TN) and Doug Melton (Harvard University, Cambridge, MA), respectively.

To activate recombination by *ElaCreERT and Ptf1a*$^{CreERT/+}$*,* we administered tamoxifen (Sigma), dissolved in corn oil, by oral gavage. Acute pancreatitis was induced by caerulein treatment, following our established protocol in which mice received repeated intraperitoneal (i.p.) injections of caerulein (Bachem; 0.1 µg/g in 0.8% NaCl) eight times daily over 2 days (16 injections total) [3,25].

Tissue processing and staining

Mice were euthanized with isoflurane, and tissues were dissected into ice-cold PBS for further processing. BrdU labeling was used to identify cells in S-

phase of the cell cycle, and was administered either by injecting mice with BrdU (50 µg/g body weight) at 1 hour prior to sacrifice, or twice daily for 6 days. Pancreata were dissected and processed for paraffin or frozen sections as previously described[3]. Human samples were formalin-fixed, paraffin-embedded archival specimens of de-identified human pancreata, provided by Dr. Mary Bronner, Department of Pathology, and sectioned similarly to mouse samples. Primary antibodies used for immunostaining were: rabbit anti-EYFP (Abcam), sheep anti-amylase (BioGenesis), mouse anti-E-cadherin (Millipore), goat anti-E-cadherin (R&D systems), and rat anti-BrdU (Abcam). Secondary antibodies raised in donkey were obtained from Jackson ImmunoResearch.

Whole pancreas digestion for cytospin

Samples processed for whole pancreas digestion were dissected into ice-cold 1X Hank's Balanced Salt Solution (HBSS, Mediatech) and minced with scissors into smaller fragments. The sample was then briefly centrifuged (600 rpm, 5 min), resuspended in 0.02% Trypsin-EDTA (Gibco/Life Technologies) in PBS, and incubated in a shaking water bath at 37^{o}C for 15 min (reduced to 10 min for mice less than 1 month old). Digestion was terminated by the addition of an equal volume of Dulbecco's modified Eagles media (DMEM, Mediatech) with 10% fetal bovine serum (FBS) (referred to as DMEM/FBS) and centrifuged as before. The digested sample was resuspended and centrifuged twice in 1X HBSS with 4% bovine serum albumin (BSA) fraction V (Fisher Scientific) and 0.01% DNAse1 (Sigma) (henceforth, wash buffer). The sample was then

digested a second time in 1X HBSS with 1 mg/ml Collagenase P (Roche), 0.1 mg/ml soybean trypsin inhibitor (Sigma) and 0.2 mg/ml BSA, incubated in a shaking water bath at 37^{o}C for 1 hour. Sample was triturated and filtered through a sterile 100 μm mesh filter, pelleted by centrifugation as previously, and washed twice in wash buffer. The sample (predominantly comprising intact or partially disrupted acinar clusters) was digested for a third time in 0.02% trypsin-EDTA, incubated in a shaking water bath at 37^{o}C for 15 min (trypsin concentration reduced to 0.01% for mice less than 1 month old). Trypsin digestion was stopped by addition of DMEM/FBS as before, and the sample filtered through a sterile 40 μm mesh filter. Samples were pelleted and suspended in wash buffer three times, and cells counted after the final resuspension. Cells were diluted into 4% paraformaldehyde (PFA) in PBS at a final concentration of 60,000 cells/ml and fixed while rocking at 4°C, 15 min. For each cytospin spot, 250 μl (15,000 cells) were spun onto a slide with a (Thermo Scientific, Cytospin 4) cytospin, 500 rpm for 3 min. Slides were removed from the centrifuge, washed briefly in 1X PBS, air dried for 30 min and stored at -80^{o}C. Staining followed our standard protocol for frozen sections.

Image quantification and analysis

To determine acinar cell nuclearity, we photographed 10 independent fields (20X original magnification) per specimen, across multiple sections and cytospin spots. Samples were scored manually in Adobe Photoshop CS5 using the Analysis>Count tool function. Calculations were performed in GraphPad

(Prism) and all results are reported as mean ± s.e.m. *P*-values were calculated by two-tailed, unpaired *t*-test, or ANOVA where specified.

Images were captured sequentially on an Olympus IX71 microscope, with the DAPI images always taken first. The nuclear corrected fluorescence (NCF) was determined using ImageJ, as follows. First, the DAPI-stained image was duplicated and thresholded to produce a binary mask encompassing each nucleus. This "mask" was imposed onto the unedited DAPI image, with manual curation to delete nuclei that clearly overlap with one another or are fragmented. The ImageJ Measure function returned the area, integrated pixel density, and mean intensity for each nucleus; we also measured the mean intensity of areas lacking nuclei, to obtain mean background fluorescence. The following formula was used to calculate the NCF for each nucleus: NCF = Integrated Density – (Area of selected nuclei x Mean Fluorescence of Background Reading). We next calculated the median NCF for each field and, because the majority of nuclei are diploid, set the median NCF to represent a diploid value. We used the median NCF to normalize NCF values for each field, transforming the NCF values into multiples of the diploid number, which we call the median-corrected NCF value (MNCF). The MNCF for >10 fields are pooled to represent a single mouse.

We next grouped MNCF values for each moues into distinct ploidy classes using *k*-mean*s* clustering analysis as described here [37]. The goal of *k*-means clustering is to partition the data in an unbiased way into *k* groups or clusters with the closest means. To determine the most likely number of peaks, we used the Calinski-Harabasz pseudo F statistic to define the best stopping solution [37]. Once

the number of ploidy peaks was determined, we found the fraction of MNCF values for each peak and averaged those values for several mice for each timepoint. This analysis allows us to determine the fraction of nuclei that are 2n, 4n, and >4n for each timepoint analyzed. After assigning MNCF values to distinct ploidy peaks, we retrospectively determined whether individual nuclei were present within a mono- or binucleate acinar cell.

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CHAPTER 4

β-CATENIN IS CRITICAL FOR CAMOSTAT-INDUCED HYPERPLASIA BUT NOT HYPERTROPHY

Introduction

The adult pancreas is normally a quiescent organ but is capable of growth as an adaptive response to environmental stimuli such as increased dietary protein. One of the most established regulators of pancreatic growth is the gastrointestinal hormone cholecystokinin (CCK), which is secreted by enteroendocrine cells of the small intestine in response to increased dietary protein [1]. The stimulation of endogenous CCK with oral trypsin inhibitors such as camostat, administration of exogenous CCK, or its chemical analogue caerulein all promotes pancreatic growth in a number of animal models[1,2]. Furthermore, the genetic ablation of CCK receptors or chemical inhibition via CCK receptor antagonists blocks this trophic response [1,3].

The calcineurin and mTOR signaling pathways have been identified as downstream effectors of CCK, driving pancreas growth in response to camostat feeding [1-4]. In the camostat-induced growth model, pancreatic proliferation peaks after 2 days of feeding with the trypsin inhibitor camostat, and plateaus by 7 days, resulting in a net doubling in pancreatic mass [2]. The growth plateau that occurs

at 7 days has been associated with decrease in CCK-dependent stimulation of pancreatic protein synthesis rather than a decrease in circulating CCK hormone levels [5], as these levels remain elevated even after camostat has been removed [2]. The calcineurin and mTOR pathway have received considerable attention, with respect to camostat-induced growth, as the inhibition of these pathways abolishes the camostat-dependent increases in pancreas mass, DNA content, and BrdU incorporation[2-7]. However, these pathways do not promote perpetual growth, as their activity is reduced with prolonged camostat feeding [3,6,8]. Together, these studies suggest that pancreas growth following camostat feeding is not limited by the intrinsic capacity of acinar cells to proliferate, but instead by the ability for various pathways to remain activated and thus sustain acinar cell proliferation [5].

CCK levels decline after weaning in rodents, making this gut hormone a strong candidate for the age-dependent reduction in acinar cell proliferation [9], [10]. Additionally, in camostat-fed CCK-deficient mice, increased acinar cell proliferation is abolished, as assayed by incorporation of the S-phase marker BrdU[3]. However, in response to a high protein diet, the pancreatic weight of CCK-null mice increases proportionally with controls[3]. Therefore, the primary function of CCK in pancreas growth appears to be increased cell number and not increased cell size, while CCK-independent mechanisms exist to drive organ hypertrophy even when cell proliferation remains low.

Previous studies by our lab have determined that the Wnt signaling component, β-catenin, is continuously required for acinar cell proliferation in the

postnatal pancreas [11]. Our lab has also shown that activation of β-catenin is sufficient to increase pancreas growth early in postnatal life, but that this is eventually overridden by an age-dependent decline in acinar cell proliferation (Chapter 3). We therefore hypothesize that β-catenin acts like a rheostat to amplify the proliferative input from other sources, and is not itself driving acinar cell proliferation during postnatal growth. Accordingly, the decline in acinar cell proliferation with age may reflect reduced growth stimuli provided by other inputs, including CCK, rather than altered sensitivity to β-catenin signaling. Therefore, the objective of this study is to determine whether β-catenin is required for CCK-dependent growth, establishing this pathway as a broad regulator of acinar cell proliferation in the pancreas.

Results

Pancreatic mass increases with camostat feeding in ABKO mice

Previous studies in mice have shown that pancreatic mass doubles after 7 days of camostat feeding in tandem with an increasing total DNA and protein content [1,2]. The camostat-induced doubling in pancreas mass is considered to be driven largely by increased cell proliferation, though increased mass can still occur independent of proliferation in CCK-null mice driven by increased hypertrophy [2,3]. Our lab has shown that the deletion of β-catenin significantly reduces acinar cell proliferation during postnatal and regenerative growth [11]. However, it is not known whether β-catenin is required for camostat-dependent

acinar cell proliferation, and therefore could impact camostat-dependent increases in pancreas mass.

We generated control and acinar-specific β-catenin knockout (ABKO) mice as previously described, using the acinar-specific, tamoxifen-inducible *Elastase-CreERT* deletor strain [11]. Mice were raised to 6-8 weeks of age before receiving tamoxifen (10 mg) to induce β-catenin deletion and concomitant labeling with an EYFP reporter ($R26R^{EYFP}$), and allowed a further 7-day recovery period prior to camostat feeding. Camostat (FOY-305) was added to powdered food (0.1% by weight) and fed to mice twice daily (morning and evening) throughout the experimental period [4]. Both control and ABKO mice were sacrificed after 2 or 7 days of camostat feeding, together with chow-fed controls.

We have previously shown that relative pancreatic mass is maintained from weaning to at least two years of age at ~1% of total body weight (Chapter 3). After 2 days camostat feeding, at which point acinar cell proliferation should be at its peak [7], we observed a small increase in pancreas mass for both control and ABKO pancreata (Figure 4-1A). By 7 days of camostat feeding, relative pancreas mass had approximately doubled in both control and ABKO mice (n=5-6 mice per treatment group) (Figure 4-1B). Interestingly, there was no significant difference in relative pancreas mass between control and ABKO mice fed either chow or camostat for 7 days.

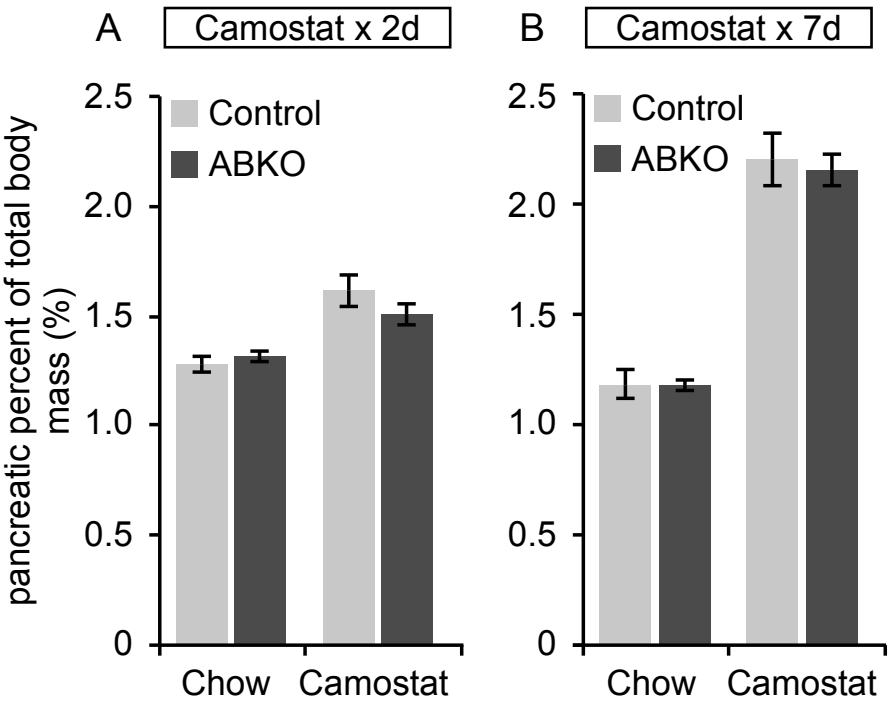

Figure 4-1. Camostat feeding increases pancreas mass. For all our experiments, adult chow-fed mice had pancreata that were ~1% of total body weight (relative pancreas mass). (A) After 2 days of camostat feeding, relative pancreas mass increases slightly for both control and ABKO mice. (B) After 7 days of camostat feeding, pancreas mass has approximately doubled in both control and ABKO mice.

β-catenin is critical for CCK-dependent acinar cell hyperplasia

The adult mouse pancreas is a highly quiescent organ and very few acinar cells would be expected to divide during the course of this experiment in the absence of camostat feeding. Therefore, camostat-induced growth provides a suitable paradigm in which to test whether β-catenin is required for CCK-dependent acinar cell proliferation. By comparing identically treated control and ABKO mice, we should be able to detect a requirement for β-catenin by the relative frequency of EYFP-labeling in ABKO pancreata compared with controls, as previously described [11]. Mice were pulsed with tamoxifen (10 mg) 7 days prior to camostat feeding, a dose previously established to induce recombination in 60-80% of acinar cells in both control and ABKO mice. The relative frequency of EYFP labeling after 2 days of camostat feeding was not significantly different between control and ABKO mice (data not shown). After 7 days of camostat feeding, we observed a modest but significant reduction in the pancreatic EYFP labeling index of camostat-fed ABKO mice compared to chow-fed ABKO mice [ABKO chow-fed: 69±3% (n=4); ABKO camostat-fed: 59±3% (n=4), $P<0.05$] and no difference for control mice (Figure 4-2). This suggests that unlabeled acinar cells in ABKO mice, which retain a functional copy of β-catenin, proliferate only slightly faster than mutant cells and therefore do not dramatically "dilute" EYFP+ β-catenin-deficient cells within the acinar compartment.

Several studies have shown that acinar cell proliferation peaks after 2 days of camostat feeding, but then decreases with continued feeding [7].

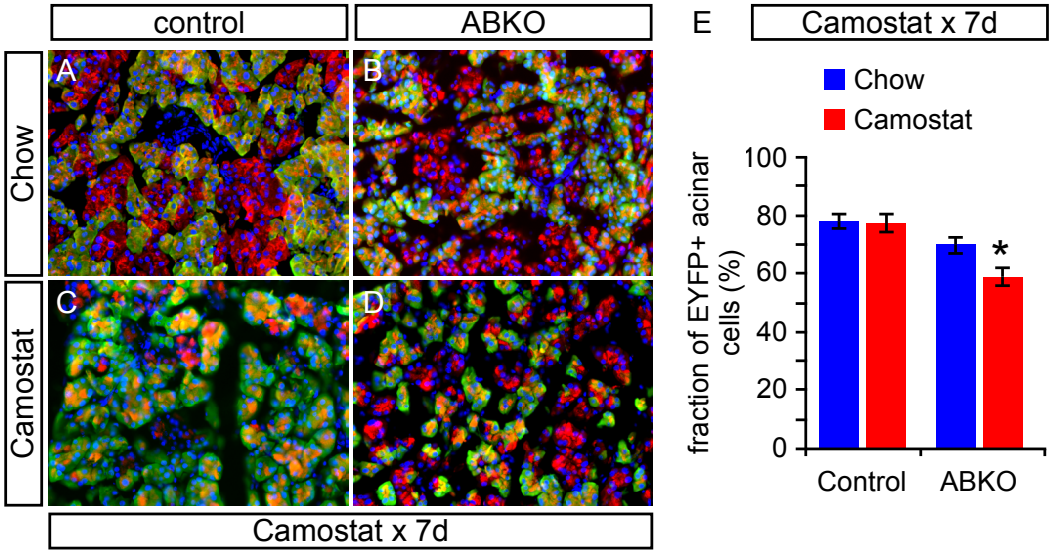

Figure 4-2. Reduced contribution of β-catenin-deficient acinar cells during camostat-induced growth. Control and ABKO mice were raised to 6-8 weeks and received a single pulse of tamoxifen (TM) 7 days prior to chow or camostat feeding. Pancreata were analyzed after 7 days on chow or camostat diet (n=5-8 mice per genotype per treatment group). (A-D) Immunofluorescence to detect EYFP lineage labeling (green) of amylase+ acinar cells (red). (E) The acinar cell EYFP labeling index is decreased specifically in camostat-fed vs. chow-fed ABKO mice, suggesting impaired proliferation relative to unlabeled cells (E, blue bars).

Additionally, in CCK-deficient mice, the mitogenic response to camostat-feeding is abolished [3]. To determine the requirement for β-catenin during this period of CCK-dependent proliferation, we analyzed control and ABKO camostat-fed mice, together with chow-fed mice, for the S-phase marker BrdU after a 1-hour pulse/chase. Immunostaining revealed a ~10-fold relative decrease in EYFP+ /BrdU+ acinar cells in ABKO mice compared with controls, 2 days after camostat feeding [control camostat-fed: 6.0±0.8% (n=3), ABKO camostat-fed: 0.53±0.25% (n=2), *P*=0.01] (Figure 4-3). Consistent with our previous study on β-catenin function in juvenile and regenerative growth [11], we found that unrecombined acinar cells (EYFP-negative, β-catenin-expressing) of camostat-fed ABKO mice do not exhibit compensatory hyperproliferation (data not shown). Therefore, despite the lack of effect on overall organ size and modest effect on EYFP labeling index, we conclude that deletion of β-catenin severely impairs camostat-induced acinar cell proliferation.

β-catenin is dispensable for camostat-induced acinar cell hypertrophy

The fact that pancreatic mass increases normally in camostat-fed ABKO mice, despite reduced proliferation within ~70% of acinar cells, implies a significant role for proliferation-independent growth, or hypertrophy. Although several studies have shown that acinar cell hypertrophy contributes to CCK-dependent growth in response to camostat feeding, none have directly measured changes in acinar cell size [1,3,5,12,13]. Instead, a correlation is made between

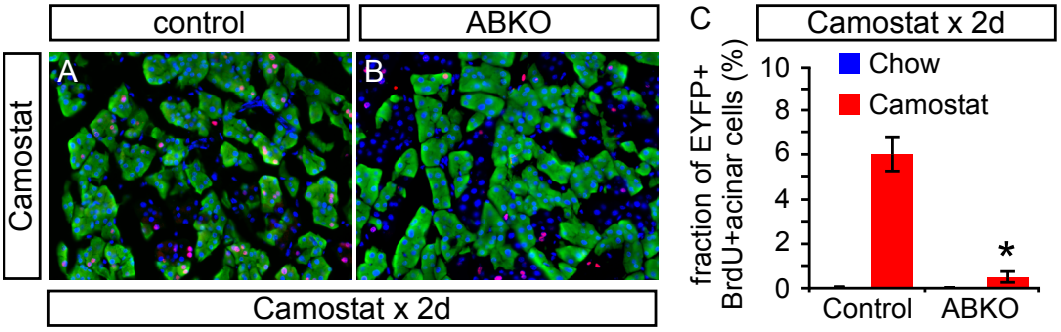

Figure 4-3. β-catenin is critical for camostat-dependent proliferation. Tamoxifen-treated mice were fed a control chow or camostat diet for 2 days, followed by a 1 hour BrdU pulse to label cells in S-phase prior to sacrifice. (A, B) Staining for BrdU uptake (red) by EYFP+ acinar cells (green) in camostat-fed control and ABKO pancreata, indicating exclusion of BrdU from labeled β-catenin-deficient cells. (C) Quantification of EYFP+ BrdU+ labeling index reveals a dramatic reduction in camostat-induced proliferation of β-catenin deficient acinar cells compared to controls (n=2-3 mice per genotype per treatment group).

increased acinar cell protein content, zymogen granule, or nuclear density in sections, to serve as a proxy for increased cell size [3]. We have developed a method to digest the pancreas to a single-cell suspension, for analysis on cytospin-prepared slides, with which we can compare relative area of individual acinar cells (Chapter 3). In this way, we can directly analyze changes in acinar cell size induced by camostat feeding, and determine whether these depend on β-catenin.

As previously reported in this thesis, acinar cell nuclearity increases with age, and in the adult >35% of all acinar cells are binucleate (Chapter 3). We have also shown that binucleate acinar cells are ~1/3 larger than mononucleate cells, by surface area (Chapter 3). Using immunofluorescence on cytospin-prepared slides we measured the surface area of EYFP-labeled cells in both control and ABKO mice, after 7 days of chow or camostat feeding. Similar to our previous findings, binucleate cells in all cases were ~1/3 larger by surface area than their genotype- and diet-matched mononucleate counterparts (Figure 4-4). For ABKO chow-fed mice, we found a slight, but significant increase (ANOVA $P<0.0001$) in the mean cell size of both mono- and binucleate cells compared to control chow-fed mice (Figure 4-4B). While the biological significance of this difference is unknown, it does indicate that acinar cells can at least maintain their size in a β-catenin-independent manner. Further, we found that camostat feeding resulted in an increase in surface area of ~1.5-fold for both mono- and binucleate cells, in ABKO as well as control mice (Figure 4-4C,D). Thus, similar to loss of CCK [3], the deletion of β-catenin did not inhibit camostat-induced acinar cell hypertrophy.

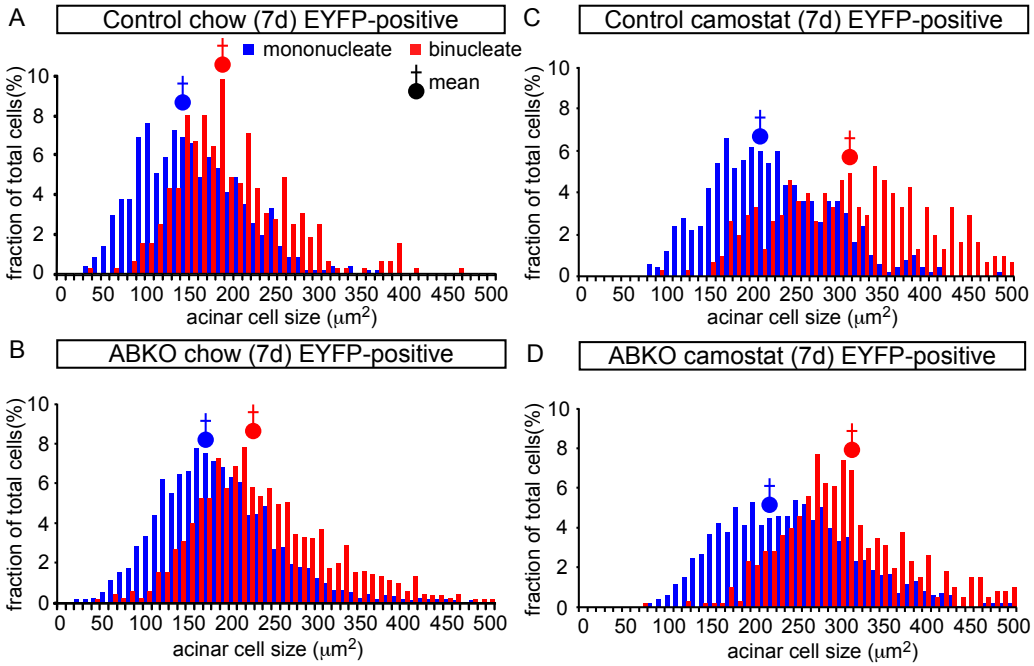

Figure 4-4. β-catenin is dispensable for camostat-induced hypertrophy. Pancreata of control and ABKO mice were digested to a single-cell suspension and analyzed as cytospin preparations after 7 days of chow (A, B) or camostat feeding (C, D). Surface area of mononucleate (blue bars) and binucleate acinar cells (red bars) were quantified separately. Regardless of genotype and treatment, binucleate cells were larger on average than mononucleate (P<0.0001 for each group).

In turn, cell growth rather than cell division may represent the major mechanism of organ mass increase during camostat feeding.

Acinar cell ploidy increases with camostat feeding

Previous studies have shown that acinar cell DNA content doubles after 7-days of camostat feeding, which has been interpreted as an increase in acinar cell numbers [3]. In this study, we show that the number of cells in S-phase after 2 days of camostat feeding is dramatically reduced in the ABKO pancreata compared to controls, yet this has no effect on pancreas mass. In addition, despite this dramatic decrease in camostat-induced acinar cell proliferation, we observed a relatively modest decrease in EYFP labeling (Figure 4-3). However, this calculation follows our previously established methodology, in which we quantify EYFP-labeled cells based on counting nuclei per field[11]. We now appreciate that a significant fraction of acinar cells are binucleate (2 x 2n nuclei), and that the division of a binucleate acinar cells would produce tetraploid mononucleate cells (1 x 4n), increasing total organ DNA content without increasing nuclear number. Thus, our ability to detect “dilution” of EYFP+, slow-dividing ABKO cells by EYFP-negative, β-catenin-expressing neighbor cells would be limited, as the division of binucleate cells would essentially go unnoticed.

To determine if such a scenario were plausible, we quantified acinar cell ploidy in control mice after 7 days of camostat feeding. We found that the proportion of tetraploid nuclei increased from <30% in chow-fed mice to >42% in

camostat fed mice (Figure 4-5). The number of >4n acinar cell nuclei is highly variable among older mice, however, we found no nuclei of higher ploidy in our control chow-fed mice. This changed with camostat feeding, increasing the fraction of >4n cells to >7% by 7-days of camostat feeding (Figure 4-5). This increase in acinar cell ploidy suggests that a significant number of binucleate acinar cells re-entered the cell cycle to produce mononucleate, tetraploid daughter cells, and thereby increasing DNA content without increasing the number of nuclei. This finding may explain why β-catenin+ cells did not appear to more dramatically outgrow β-catenin mutant cells in camostat-fed ABKO mice, despite the dramatic difference in proliferation rates between these populations. In addition, the fact that such a large number of nuclei become polyploid suggests that a diploid-to-polyploid conversion makes a significant contribution to increased DNA content during camostat feeding.

Discussion

All tissues and organs of the body are subject to the growth-regulating influences of physiologic demand, which in the pancreas limits cell turnover in the adult, but can induce adaptive growth as a response to dietary protein intake. High-protein diet feeding of mice has previously been found to increase both cell number and cell size in the adult pancreas [3]. Previous work from our lab has established β-catenin as a critical regulator of postnatal and regenerative growth, as its deletion significantly reduces acinar cell proliferation in young mice and after injury [11]. Several studies have found that camostat feeding increases

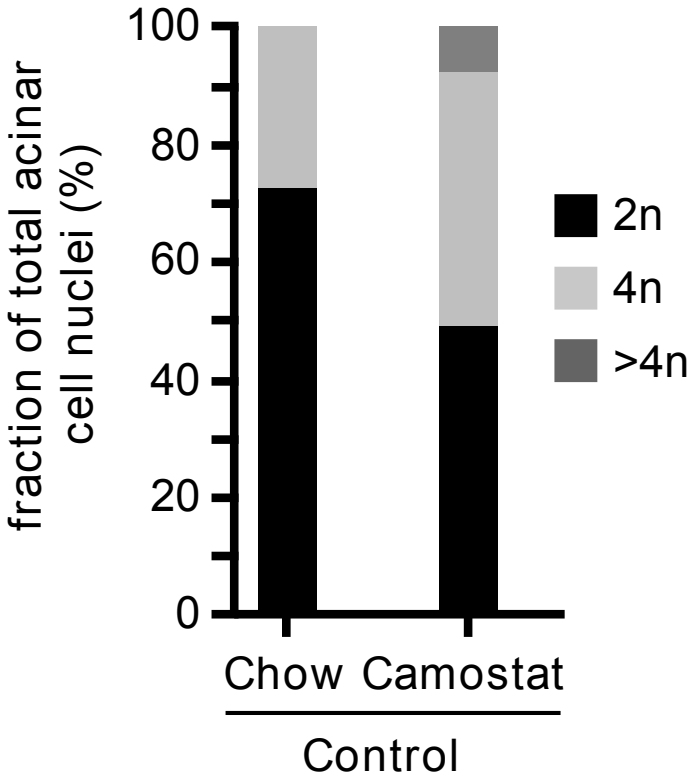

Figure 4-5. Acinar cell ploidy increases with camostat feeding. Wild-type mice were fed chow or camostat for 7 days, and analyzed for ploidy per nucleus.

proliferation in response to increased CCK-levels [2,3,14]. In this study, we have extended these findings by establishing β-catenin as critical for camostat-dependent proliferation, but dispensable for increased cell size in response to camostat-induced growth. Whether dietary protein induces β-catenin in a Wnt-dependent manner is not known. Future experiments could address the role of Wnt/β-catenin signaling in CCK-dependent growth by either conditionally deleting *Porcupine*, a gene required for proper Wnt secretion and function or by specifically deleting the signaling function of β-catenin, while leaving the proteins ability to perform its structural function intact [15,16]. Additionally, our findings support a preferential contribution of increased acinar cell size, rather than cell number, to the expansion of pancreatic mass induced by camostat. Finally, we find that a component of increased DNA content observed in other studies after camostat feeding may actually reflect diploid-to-polyploid conversion, rather than a relative increase in nuclear number. The presence of binucleate acinar cells, and their potential for division, presents an important caveat to studies of acinar cell growth in which counting of nuclei serves as a proxy for determining cell number.

We have previously determined that β-catenin is both necessary and sufficient to increase acinar cell number during postnatal growth, but this growth period is not interminable and, independent of robust β-catenin signaling, the mature pancreas displays an age-dependent decline in proliferation [9-11,17,18] (Chapter 3). Our findings supports a model where β-catenin is not itself an absolute determinant of cell cycle entry, but instead functions like a rheostat to

amplify the proliferative input from other pathways. Interestingly, the age-related decline in postnatal acinar cell proliferation coincides with a previously described role for CCK to drive increased pancreas mass during development. Therefore, it remains altogether possible that it is reduced CCK-levels that are limiting acinar cell proliferation during postnatal growth, and that β-catenin is critical to amplify this growth signal.

While camostat-induced proliferation depends on β-catenin, reduction of this proliferation does not preclude a doubling of pancreatic mass. A similar observation has been made in CCK-null mice fed a high protein diet, where pancreas mass increased similar to controls, even though acinar cell proliferation was ablated [3]. The increased mass in CCK-null mice is largely the result of mTOR-dependent acinar cell hypertrophy, and we demonstrate here, based on analysis of dissociated acinar cells, that camostat-induced hypertrophy occurs independent of β-catenin. Therefore, it is likely that acinar cell hypertrophy is the primary means by which pancreas mass increases; the ultimate physiological impact of impaired proliferation remains to be determined. Also to be determined is the potential pathological impact of increased tetraploidy induced by high protein feeding. As tetraploid mammalian cells are inherently prone to generate chromosomally-unstable offspring [19], camostat feeding may establish a pool of acinar cells with increased tumorigenic potential. Further studies are required to address the mechanisms by which CCK and β-catenin interact to regulate acinar proliferation, as well as the consequences to organ health when this interaction is activated or inhibited.

Methods

Experimental design

In all experiments, animals were raised to 6-8 weeks and administered 10 mg tamoxifen (Sigma) by oral gavage, 7 days prior to camostat feeding. Mice were acclimated to chow in powdered form for 3 days prior to further experimental manipulation. Animals were then divided into groups provided with either standard powered chow or powered chow containing 0.1% (w/w) camostat (FOY-305) for 2 and 7 days. Camostat was generously provided by Dr. John Williams (University of Michigan).

Mouse breeding and genetic manipulation

All mouse experiments were performed according to protocols approved by the University of Utah Institutional Animal Care and Use Committee. The generation of acinar specific β-catenin knock out [ABKO (*Ctnnb1*$^{\Delta/lox}$*; R26R*$^{EYFP/+}$*; Elastase-CreERT*)] mice and their sibling controls [control (*Ctnnb1*$^{lox/+}$*; R26R*$^{EYFP/+}$*; Elastase-CreERT*)] were carried out as described in Chapter 3.

Tissue processing, histology, and image quantification

Tissues were processed and stained as described in Chapter 3 of this thesis. To determine pancreatic percent of total body weight, each mouse was weighed prior to sacrifice and the harvested pancreas was independently weighed post-mortem.

To determine acinar cell EYFP labeling and BrdU staining, we photographed 10 independent fields (20X original magnification) per specimen, across multiple sections. Samples were quantified using ImageJ additive image overlay, as described in Chapter 2 of this thesis [11]. Images where the markers do not overlap were scored manually in Adobe Photoshop CS5 using the Analysis>Count tool function, as previously described in Chapter 3 of this thesis. Acinar cell ploidy was determined quantification of nuclear DAPI fluorescence and *K*mean*s* cluster analysis, as previously described in Chapter 3 of this thesis. Calculations were performed in GraphPad (Prism) and all results are reported as mean ± s.e.m. *P*-values were calculated by two-tailed, unpaired *t*-test or ANOVA where specified.

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CHAPTER 5

SUMMARY AND CONCLUSIONS

The emphasis of my PhD thesis has been to determine whether the Wnt signaling component β-catenin and acinar cell nuclearity regulate pancreas organ size, so that they may be exploited in our understanding of diseased states. I have specifically focused my research on the role of β-catenin and the cellular process of binucleation in acinar cells during postnatal and regenerative pancreas growth. The role for β-catenin during postnatal and regenerative growth were previously unknown. Similarly, the presence and functional significance of binucleate acinar cells in the mouse and human pancreas had not been characterized. This dissertation addresses the integration of these two components of organ growth, which both promote and limit acinar cell proliferation.

β-catenin is critical for postnatal and regenerative growth

β-catenin signaling has been shown to promote growth and differentiation of acinar cells during embryonic development; however, its role in the postnatal pancreas has received considerably less attention. To address this, we deleted

β-catenin specifically in acinar cells at birth and in the adult prior to injury, while simultaneously labeling these cells with an EYFP reporter, and measured the contribution of EYFP-labeled cells to postnatal and regenerative growth. Consistent with its role in the embryonic pancreas, β-catenin was critical for acinar cell proliferation and, therefore, EYFP-labeled β-catenin deficient acinar cells were dramatically reduced (Chapter 2). Despite the high amount of β-catenin protein present at the acinar cell membrane, where one might expect it to play a structural role in cell-cell adhesion, we did not observe any defects in acinar cell integrity, organization, or identity after its deletion. Taken together, we conclude that the loss of β-catenin causes a dramatic reduction in acinar cell proliferation, but intriguingly, not a complete loss, as few β-catenin deficient acinar cells stain for markers of the cell cycle. We have incorporated this point, that β-catenin is not absolutely required for proliferation, into our rheostat model for β-catenin function in acinar proliferation described below.

Conversely, the conditional stabilization of β-catenin in ABGOF mice at birth significantly increased pancreas mass, driven by acinar cell hyperplasia (Chapter 3). The fact that ABGOF produces a reciprocal phenotype to our previously reported ABKO mice supports a key role for Wnt signaling per se in acinar proliferation. Though stabilized β-catenin is sufficient to increase pancreas mass early in postnatal life, the conditional activation of β-catenin after weaning does not. We determined that this was because acinar cells exhibited an age-dependent decline in proliferation, independent of β-catenin status. We determined that targets of β-catenin like *axin2* and *c-myc* were still highly

expressed in the adult ABGOF pancreas (data not shown). Therefore, it is not likely that β-catenin signaling is being shut down, but instead, we hypothesize that perhaps a co-regulator of acinar cell proliferation is reduced in an age-dependent manner.

Since β-catenin could not sustain interminable growth, we hypothesized that β-catenin activity was not itself an absolute determinant of cell cycle entry, but instead functioned like a rheostat to amplify the proliferative input from other pathways. Perhaps the best-characterized trophic factor during postnatal pancreas growth is the gut hormone cholecystokinin (CCK). CCK is secreted in response to the presence of protein in the small intestine [1]. Several studies have shown that endogenous CCK stimulation or exogenous introduction of CCK both increase pancreas mass in a dose-dependent manner [1,2]. More recent studies have determined that the primary means by which CCK increases pancreas mass is via increased acinar cell proliferation [3,4]. To test our rheostat model, we asked whether β-catenin was required for CCK-dependent growth, and whether β-catenin function could "tune" the proliferative input of a well-characterized acinar cell mitogen.

β-catenin is critical for camostat-dependent acinar cell proliferation

The goal of our study was to determine whether β-catenin is required for acinar cells proliferation, induced by physiological demand. To elevate circulating CCK, we fed mice the protease inhibitor camostat. Camostat has been shown to

dramatically increase acinar cell proliferation in a CCK-dependent manner [4]. Camostat prevents protein digestion; therefore, the body reacts by increasing acinar cell enzyme secretion as if there was and excess of dietary protein needing to be digested. We found a 10-fold reduction in BrdU+ acinar cells at 2 days after camostat feeding, compared to controls, indicating that β-catenin is critical for camostat-induced acinar cell proliferation (Chapter 4). Interestingly, although acinar cell proliferation was significantly reduced in the ABKO mice, their pancreata doubled in mass, similar to control camostat-fed mice. To address this discrepancy, we developed a single cell assay to analyze changes in acinar cell surface area in response to camostat feeding. We hypothesized that hypertrophy was enhanced in the ABKO pancreas compared to controls and therefore made up for increased pancreas mass. However, we found that for both control and ABKO pancreata, acinar cell surface area significantly increased with camostat feeding by ~1.5-fold, compared to chow-fed controls for both genotypes. Thus, camostat-induced acinar cell hypertrophy does not require β-catenin, but increased hypertrophy cannot account for the increase in ABKO pancreas mass despite reduced proliferation.

To assess the relative contribution of β-catenin deficient acinar cells to camostat-dependent growth, we employed the same EYFP reporter labeling strategy as described previously (Chapter 2, and see above). However, this only added to the complexity, where after 7 days camostat feeding, the contribution of EYFP labeled β-catenin deficient acinar cells in the ABKO mice were only modestly reduced compared to controls. This required us to rationalize how a 10-

fold reduction in BrdU+ EYFP+ acinar cells in camostat-fed ABKO mice could translate into only a slight reduction in EYFP+ cell number. We realized that our measurements of EYFP+ cell numbers were based on counts of nuclei, following a protocol that we developed prior to our appreciation that nucleus number and DNA content was nonuniform among acini (Chapter 3, and see below). If binucleate cells (2 x 2n nuclei) were to divide to give rise to mononucleate, tetraploid daughter cells (1 x4 n), this would not result in a net increase of nuclei, and would result in our underestimating the extent to which ABKO cells were "diluted out" by unlabeled wild-type cells, despite their dramatically reduced proliferation. In fact, we found that the number of 4n and >4n cells significantly increased in wild-type mice after camostat feeding, suggesting that a significant number of S-phase events in control pancreata result in an increase in ploidy without an increase in nucleus number. This finding may reconcile our seemingly contradictory BrdU and EYFP data, as the loss of β-catenin-dependent cell cycle entry would not necessarily translate into a decrease in EYFP-labeled acinar cell nuclei. Our results also suggest that acinar cell hypertrophy, which is β-catenin-independent, is the major driver of increased organ mass in response to camostat feeding.

Regulated changes in acinar cell DNA content during postnatal growth

Although binucleate acinar cells have been characterized in the rat pancreas, their existence in mouse is less well described and, for the human

pancreas, their presence is completely undescribed. Our lab anecdotally knew of the presence of binucleate acinar cells, as they are easy to see in histological section. However, it was not until their presence confounded our quantification of cell size in sections that we began to take note of not only their abundance, but also their functional significance. We wondered if binucleate cells represented a terminally differentiated state, which could explain the age dependent decline in acinar cell proliferation we observed in our postnatal β-catenin studies. To address the role of this cell type in the pancreas, we established a timecourse to characterize the generation and steady state maintenance of binucleate acinar cells in the adult pancreas (Chapter 3). We found that during the suckling-to-weaning transition (P17-P21), binucleate acinar cells emerged and increased in number to comprise >35% of all acinar cells in the mature pancreas. This proportion remains constant in the mature pancreas from 1 month to 2 years of age in the absence of injury. We are also the first study to describe the presence of binucleate acinar cells in the human pancreas where they contribute to >12% of all acinar cells.

Interestingly, we only observed mononucleate acinar cell contributing to juvenile growth, as determined by BrdU uptake prior to P30, but after this timepoint, we found mono- and binucleate acinar cells entering the cell cycle at nearly the same frequency. Thus, we find two regulated transitions in acinar cell DNA content: first, acinar cells transition from almost uniformly complete cytokinesis to a significant frequency of incomplete mitosis, generating binucleate cells at weaning. These cells are initially inhibited from proliferating, even while

overall cell division rates are relatively high, but then from 1 month of age are able to re-enter the cell cycle.

The observation that binucleate cells could re-enter the cell cycle in the mature pancreas is similar to what is observed for the liver, where hepatocyte ploidy increases via a binucleate intermediate, to produce tetraploid mononucleate cells. We hypothesized that because the pancreas shares a common developmental origin with the liver, acinar cells too could be increasing their ploidy through a binucleate intermediate. To test this, we developed an assay to both quantify acinar cell ploidy on a cell-by-cell basis and statistically model the ploidy of acinar cells (Chapter 3). Using this technique, we found that >30% of all acinar cell nuclei were tetraploid in the mature pancreas, a frequency in agreement with prior studies based on flow cytometry of isolated nuclei [5]. These findings suggests that, similar to hepatocytes, acinar cells transition through a binucleate intermediate to produce tetraploid mononucleate cells, and thus increase acinar cell ploidy in the mouse.

Acinar regeneration is driven by the selective division of mononucleate acinar cells

Our finding that only mononucleate acinar cells contributed to juvenile growth identifies an unexpected heterogeneity in proliferation potential among acinar cells. To determine whether this heterogeneity affects the ability of acinar cells to contribute to regenerative growth, we injured the pancreas of CD-1 wild-type mice with the secretagogue caerulein, to induce acute pancreatitis. We

found that at 2 days after injury, when regenerative acinar cell proliferation is at its peak, only mononucleate acinar cells were in the cell cycle as indicated by BrdU uptake. Accordingly, at this timepoint, the number of mononucleate acinar cells was also significantly increased, suggesting that the proliferation of mononucleate cells drives an increase in mononucleate daughter cells to replace those damaged by injury (Chapter 3). The observed increase in mononucleate acinar cell number is only transient, as the pre-injury distribution of mono- and binucleate cells is restored by 7 days post-caerulein and maintained afterwards. Our finding that only mononucleate acinar cells proliferate during regeneration establishes a new proliferative heterogeneity within the acinar cell lineage, as well as suggesting the existence of a previously undescribed mode of cell cycle control that can distinguish cells based on nuclearity.

In the adult pancreas, the reversion to mononuclear only proliferation during regeneration is critical to maintain acinar cell ploidy: if both mono- and binucleate acinar cells re-entered the cell cycle during periods of high proliferation, such as injury, this would produce a high number of tetraploid cells. Tetraploid cells have an extra set of centrosomes, which would likely confound mitosis if they were to re-enter the cell cycle, thereby producing an aneuploid cell. However, we do not observe an increase in acinar cell ploidy, presumably by the exclusion of binucleate acinar cells from the cell cycle. This is different from our previous findings in camostat-fed mice, where increased BrdU uptake significantly increases acinar cell ploidy. This suggests that perhaps in the adult pancreas, adaptive organ growth is not a generalized recapitulation of postnatal

growth, but a specific response to the stimulus, as is evidenced by increasing cell number for regenerative growth and increasing cell ploidy for camostat-dependent growth. For regeneration, the restriction of binucleate acinar cells from the cell cycle is only transient, however, and by 14 days post-caerulein treatment, binucleate acinar cells again re-enter the cell cycle at the same frequency as in the uninjured pancreas. By this stage, however, the overall proliferation rate has declined considerably, so that binucleate cell division will add relatively little to the burden of polyploid acinar cells. Therefore, the reversion to mononucleate only proliferation in the adult pancreas during regeneration protects the pancreas from increasing levels of aneuploid-vulnerable cells.

Acinar cell nuclearity, ploidy, and cancer

Previous work from our lab and others have shown that the genetic and morphologic events that will give rise to pancreatic ductal adenocarcinoma (PDAC) arise in the acinar cell lineage, identifying this cell type as the cell of origin in pancreatic cancer [6,7]. To this end, we have characterized the changes in acinar cell nuclearity and ploidy during normal pancreas development with the hope of identifying a population of cells that may be predisposed to malignant transformation. Of particular interest is the population of mononucleate tetraploid (1 x 4n nuclei) acinar cells with a duplicated set of centrosomes, in the adult pancreas. Many tumors, including those of pancreatic origin, show centrosome aberrations, indicating an underlying deregulation of centrosome duplication or

segregation [8-10]. The risk of generating a cell with an altered number of centrosomes is that a subsequent mitosis will produce an aneuploid cell.

Aneuploidy provides a mechanism by which an acinar cell could gain or lose chromosomes in the stepwise progression toward tumorigenesis. This is a key mechanism for tumor suppressor loss of heterozygosity (LOH), which affects pancreatic cancer as well as other malignancies. Indeed, pancreatic cancers frequently exhibit greater than diploid chromosome content, suggesting an origin from polyploid cells. Pancreatic cancer is known to depend on activation of the oncogene *KRAS* and loss of tumor suppressor genes such as *P53*, but whether these mutations arise in mono- or binucleate acinar cells has any bearing in the stepwise progression toward cancer is not known. We tested whether these mutations could alter pancreatic nuclearity or ploidy in adult mice, but did not observe changes in either the frequency of binucleate cells or the generation of tetraploid cells. While mutations in *KRAS* and *p53* did not directly alter acinar cell DNA content, with age, the susceptibility of tetraploid acinar cells to undergo chromosomal gain and loss could contribute to copy-number changes that may predispose these cells to tumorigenesis. It is therefore important for future studies addressing the cell of origin in pancreatic cancer to include the analysis of nuclearity and ploidy, as they may more precisely define the mechanism for malignant transformation.

The implications of increased DNA content for pancreas research

One of the goals of studying acinar cell nuclearity was to characterize the genesis and steady state frequency of binucleate cells during postnatal and regenerative growth, with the idea that we might find a distinct class of cells that was predisposed to oncogenic transformation. However, the genomic heterogeneity of acinar cells by themselves may complicate this genetic analysis. For example, Cre recombinase-mediated deletion of genes flanked by loxP sites (floxed) for a mononucleate cell presents only two outcomes, either delete the floxed allele or not, depending on the concentration and duration of Cre expression. However, for a binucleate cell, it is theoretically possible to delete the floxed allele in one nucleus, leaving a copy intact in the other nucleus. Adding to the complexity, the same is true for recombination of a lineage label in the same cell, making it entirely possible to only delete the floxed knockout allele in one nucleus, while activating the lineage label in one or both nuclei. This would result in heterogeneity among lineage labeled cells, such that a subset may retain protein expression from the floxed knockout allele due to incomplete deletion between the two nuclei. This scenario illustrates the Achilles heel of performing conditional mouse genetics in binucleate and polyploid cells. However, for our ABKO studies addressed in Chapter 2 and 4, this was less of a problem because we could easily detect the deletion of β-catenin with antibodies. Therefore, having a good antibody to detect deletion in combination with a reporter is essential.

This could have additional consequences for tumor modeling in mice: a 2 x 2n binucleate cell or a 1 x 4n mononucleate tetraploid cells will have extra copies of tumor suppressor genes compared to 1 x 2n mononucleate diploid cells. On the other hand, the propensity of tetraploid cells to generate chromosomally unstable offspring means that extra tumor suppressor genes might be rapidly shed, such that a cell with one mutant copy of *p53* and three wild-type, for example, might be more prone to tumorigenesis than a cell with one mutant and only one wild-type. Taken together, increased DNA content either by increased nuclear number or nuclear ploidy presents both practical and analytical problems that will need to be addressed in the field of mouse pancreas genetics.

Conclusions

The work in this dissertation demonstrates that acinar cell proliferation, nuclearity, and ploidy are controlled by the signaling protein β-catenin as well as by novel intrinsic and extrinsic signaling factors, essential for postnatal and adaptive growth. This work has established the role for β-catenin not as a mitogen itself, but as a rheostat to tune the proliferative input from other sources. Additionally, we have generated the most comprehensive characterization of acinar cell nuclearity and ploidy in mouse pancreas to date, as well as established the relevance of acinar nuclear number to the human pancreas. It is my hope that this work will contribute to a better understanding of the physiological mechanisms controlling pancreatic organ size, and will provide insight into the origin and nature of diseases that affect acinar cell proliferation.

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APPENDIX

β-CATENIN SIGNALING IS CRITICAL FOR PROLIFERATION AND EXPANSION OF ACINAR CELLS DURING POSTNATAL GROWTH

Results

We have previously shown, via conditional knockout approaches, that β-catenin is critical for proliferation of acinar cells during postnatal and regenerative growth of the pancreas [1]. However, β-catenin plays key roles in both Wnt signaling and cell-cell adhesion, and therefore presents a caveat for our experiments in assigning the function of this protein to a specific pathway. Recently, a β-catenin signaling deficient allele was generated by the combination of a point mutation in the fourth exon (D164A) and a truncation in the C-terminus, ablating the signaling function while maintain the structural function of the gene, and is referred to in this thesis as double mutant or DM (*Ctnnb1*^{DM}) [2]. To generate an acinar cell-specific β-catenin signaling-deficient mice (ABsKO) and their sibling controls, we crossed male mice heterozygous for a signaling-deficient allele and floxed allele of β-catenin that also carry an acinar cell-specific Cre-driver (*Ctnnb1*$^{DM/lox}$; *ElastaseCreERT*) to females homozygous for the *R26R*^{EYFP} reporter and the floxed allele of β-catenin (R26R$^{EYFP/EYFP}$; *Ctnnb1*$^{lox/lox}$).

Importantly, both the ABsKO mice, as well as controls expressing *ElastaseCreERT* alone, were established with a *R26R*^{EYFP} reporter construct in the background, permitting tamoxifen-inducible EYFP labeling of recombined acinar cells [1,3]. By comparing EYFP-labeling of acinar cells among identically treated control and ABsKO mice, we should detect the effect that proliferation has on binucleation during postnatal development. To conditionally delete the signaling function of β-catenin in mice, we administered 10 mg tamoxifen to nursing females at birth (P0) and analyzed EYFP labeling after a "chase" period of 7 or 30 days. As previously described [1], we found widespread EYFP expression by acinar cells of all genotypes. Mice were administered a 1-hour pulse of BrdU (50μg/g) prior to sacrifice to label cells in S-phase. ABsKO mice administered tamoxifen at birth and sacrificed at P7 had a significant reduction in BrdU+ EYFP+ acinar cells compared to controls [control: 3.5±0.18% n=3, ABsKO: 1.8±0.2% (n=3), P<0.004%] (Figure A-1). This finding is consistent with our previous findings in ABKO mice, suggesting that the signaling function of β-catenin is critical for acinar cell proliferation during postnatal growth [1]. By P30, the fraction of EYFP-labeled acinar cells was significantly reduced in ABsKO mice compared to controls [control: 36.5±3.8% (n=6), ABsKO: 10.4±1.2% (n=8), P<0.0001] (Figure A-2). Therefore, during postnatal pancreas growth, the signaling function of β-catenin is critical for acinar cell proliferation to expand acinar cell number.

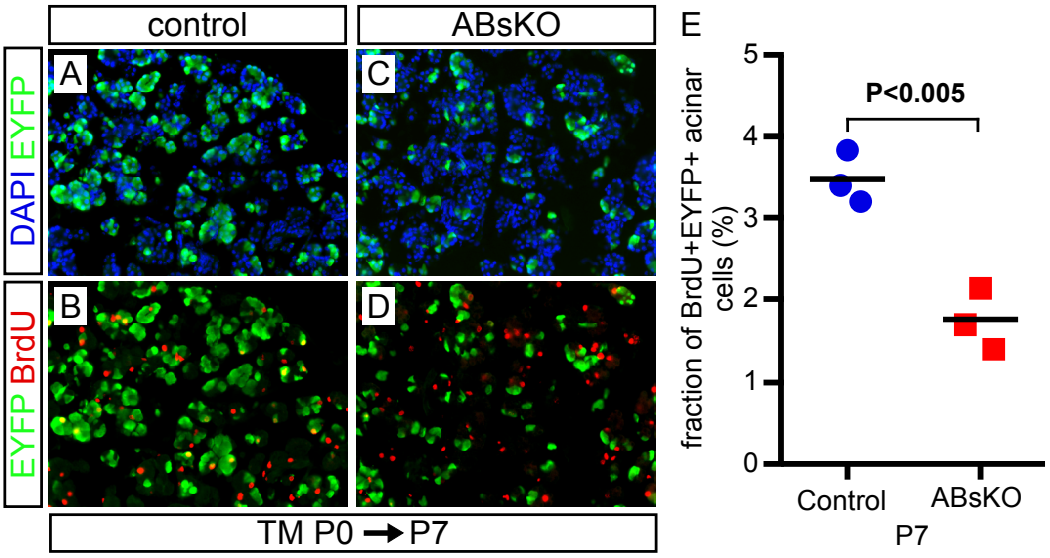

Figure A-1. β-catenin signaling is critical for acinar cell proliferation during postnatal growth. To determine whether β-catenin signaling is required for acinar cell proliferation in the neonatal pancreas, we pulsed mice with tamoxifen on the day of birth, via maternal gavage, and analyzed neonatal pancreas (P7). Prior to sacrifice, mice received a 1-hour pulse of BrdU to label proliferating acinar cells. (A-D) Immunofluorescent staining for EYFP+ acinar cells (green) and DAPI stained nuclei (blue) labeled by the S-phase marker BrdU (red). (B, D) The fraction of proliferating EYFP+ cells, marked by BrdU antibody labeling is markedly decreased in ABsKO mice compared to controls. (E) Quantification of the BrdU labeling index of EYFP+ cells reveals a significant reduction in the proliferative capacity of β-catenin signaling deficient acini (n=3 mice per genotype).

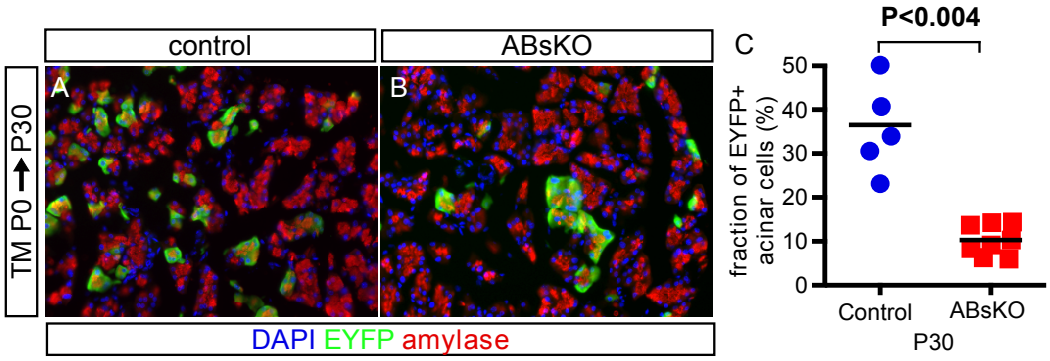

Figure A-2. β-catenin signaling is critical for acinar cell expansion during postnatal growth. Control and ABsKO mice were given a single dose of tamoxifen (TM) at birth, and analyzed as juveniles (P30). (A, B) Immunofluorescence to detect EYFP lineage labeling (green) of amylase+ acinar cells (red), reveals fewer EYFP+ cells in ABsKO pancreata compared to controls. (C) The EYFP labeling index is significantly decreased for ABsKO mice (red) at P30 compared to controls (blue).

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